PROLONGED HEAT STRESS OF *LACTOBACILLUS CASEI* GCRL163 AND THE IMPACT ON THE CELL PHYSIOLOGY AND PROBIOTIC FUNCTIONALITY USING PROTEOMICS

By

Adu Kayode Titus, B.Sc. M.Sc. GradCertRes.

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This thesis includes work which has been published in a peer-reviewed journal. Proportionate co-author contributions were as follows:

Chapter 3: Adu Kayode Titus (60%), Richard Wilson (10%), David Nichols (5%), Anthony Baker (5%), John P. Bowman (5%), Margaret L. Britz (15%).

Chapter 4: Adu Kayode Titus (65%), Richard Wilson (5%), Anthony Baker (5%), John P. Bowman (5%), Margaret L. Britz (20%).

Chapter 5: Adu Kayode Titus (70%), Anthony Baker (5%), John P. Bowman (5%), Margaret L. Britz (20%).

Chapter 6: Adu Kayode Titus (65%), Anthony Baker (5%), John P. Bowman (5%), Margaret L. Britz (25%).

Chapter 7: Adu Kayode Titus (65%), Anthony Baker (5%), John P. Bowman (5%), Margaret L. Britz (25%).

Details of the Author roles:

- Mr. Adu Kayode Titus (candidate) made key contributions to experimental design, execution of experimental work, data analysis as well as the writing of the manuscripts.
- Dr. Richard Wilson from the Proteomic Units, Central Science Laboratory, University of Tasmania (Australia) contributed to proteomic and bioinformatic data analyses
- Dr. Anthony Baker from the Tasmanian Institute of Agriculture, University of Tasmania, Hobart, Tasmania (Australia) provided directional advice as supervisor and contributed to experimental design.
- Dr David Nichols from the Central Science Laboratory, University of Tasmania (Australia) contributed to the fatty acid analysis in Chapter 3.

- Assoc. Prof. John P. Bowman from the Tasmanian Institute of Agriculture, University of Tasmania, Hobart, Tasmania (Australia) provided directional advice as supervisor and contributed to the bioinformatic and statistical data analysis.
- Prof. Margaret L. Britz, from the Tasmanian Institute of Agriculture, University of Tasmania, Hobart, Tasmania (Australia) provided directional advice as primary supervisor and contributed to the experimental design, general review of the analyses, protein structural modelling in Chapter 3, manuscript preparation and research program implementation.

We, the undersigned, agree with the above details on the proportion of work undertaken for each of the above chapters or submitted peer-reviewed manuscript as contributions to this thesis:

Prof. Margaret L. Britz
Primary supervisor
Tasmanian Institute of Agriculture
University of Tasmania
Hobart, Tasmania (Australia)

Prof. Holger Meinke
Director
Tasmania Institute of Agriculture
University of Tasmania
Hobart, Tasmania (Australia)

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ABSTRACT

Lactic acid bacteria (LAB) are exposed to several potential stressors during probiotic formulation, manufacture of fermented food and passage through the gastrointestinal tract (GIT). To understand the physiological and molecular mechanisms through which *L. casei* GCRL163 adapts to persistent elevated temperature, label-free quantitative proteomics of cell-free extracts was used to characterize the global responses of the strain cultured anaerobically in bioreactors at 30 to 45°C, pH 6.5, together with GC-MS for fatty acid methyl ester analysis at different growth phases. At higher growth temperatures, repression of energy-consuming metabolic pathways, such as fatty acid, nucleotide and amino acid biosynthesis, was observed, while PTS- and ABC-type transporter systems associated with uptake of nitrogen and carbon sources were up-regulated. Alkaline shock protein Asp23_2 was only detected at 45°C, expressed at high abundance, and presumptive α-L-fucosidase only at 40 and 45°C, with highly increased abundance (log_2-fold change of 7) at 45°C. We identified a novel SecB homolog as a protein export chaperone putatively involved in post-translational translocation systems, which was down-regulated as growth temperature increased and where the modelled 3D-structure shared architectural similarities with the *Escherichia coli* SecB protein. Membrane lipid analyses revealed temporal changes in fatty acid composition, cyclization of oleic acid to cyclopropane and novel cyclopentenyl moieties, and reduced synthesis of vaccenic acid, at higher temperatures. An 18kDa α-crystallin domain, Hsp20 family heat shock protein was more highly up-regulated in response to heat stress compared to other molecular chaperones, suggesting this protein could be a useful biomarker of prolonged heat stress in *L. casei* GCRL163.

Lactobacillus casei strains are non-starter lactic acid bacteria which improve cheese ripening and may interact with host intestinal cells as probiotics, for which cell surface

proteins play a key role. To understand the impact of prolonged heat stress on *L. casei* surface proteins, three complementary methods (trypsin shaving [TS], LiCl-sucrose extraction [LS] and extracellular culture fluid [ECF] precipitation) were employed to obtain proteins and peptides from *L. casei* GCRL163 cells, grown in bioreactors under controlled conditions. Label-free quantitative proteomics was used to characterize the surface heat stress response. A total of 416 proteins, including 300 extra-cytoplasmic and 116 cytoplasmic proteins, were quantified as surface proteins. LS caused significantly higher cell lysis as growth temperature increased. However, most extra-cytoplasmic proteins were exclusively obtained from LS fractions, demonstrating the utility of LiCl extraction of surface proteins in a species producing no S layer. Cell wall hydrolases, adhesins, and homologues of major secreted *L. rhamnosus* GG proteins Msp1/p75 and Msp2/p40, were up-regulated in surface and secreted protein fractions, suggesting that cell adhesion may be impacted by heat stress. Hydrophobicity analysis indicated hydrophilicity was enhanced at sub- and supra-optimal growth temperatures. The binding-capacity of *L. casei* GCRL163 to human colorectal adenocarcinoma HT-29 cells also increased for heat-stressed relative to acid-stressed cells. This study demonstrates that prolonged heat stress influences cell adhesion and abundance of surface proteins, which may impact probiotic functionality.

Understanding the expression of proteins associated with the cell surface at different growth phases can provide an in-depth insight into how bacteria manipulate their physiological processes to adapt under different environmental conditions. To this end, LFQ proteomics was applied to investigate *L. casei* GCRL163 surface proteins extracted by TS and LS at mid-exponential and stationary growth phases and also to profile the secreted ECF proteins. Our findings revealed an increased expression of some cell wall hydrolases, including BN194_23630 and SLAP domain-containing NlpC/P60 protein BN194_02820, and

putative autolysins, suggesting induced cell wall hydrolysis, peptidoglycan autolysis and exopolysaccharide degradation in stationary phase. Proteins involved in sugar and peptide uptake, including oligopeptide ABC-type transporters OppA, OppA_2 and OppD_2, and phosphotransferase system proteins FruA_3 and BglP were more abundant, with GlnA and CysK induced at stationary growth phase in different protein fractions. Transcriptional regulator LytR, involved in sensory transduction and cell wall metabolism regulation, and proteins associated with cell surface structure synthesis, including exopolysaccharides such as RmlB, RmlB_2, SpsK and SpsK _2, were more abundantly expressed at stationary phase. The abundance of several proteins linked to probiotic functionality, including moonlighting proteins (Gap, Fab_2), Tig and cell wall hydrolase NlpC/P60 protein BN194_02820, suggested that host-cell interactions would be heightened at stationary growth phase. These findings establish the growth-phase dependent change in the surface sub-proteome of *L. casei* GCRL163 that possibly promotes cell adaptation and enhanced functionality.

In addition, the regulation of prolonged heat stress response (PHSR) is important for adaptive responses that adjust cellular and molecular functions to maintain energy production and structural integrity of macromolecules and enzymes during prolonged heat stress. To investigate the induction of the regulatory proteins involved in PHSR in *L. casei,* a forensic analysis was performed on LFQ proteomic datasets of the CFEs, cell surface-associated (TS and LS extracts) and ECF protein fractions of *L. casei* GCRL163, cultured anaerobically at pH 6.5 in bioreactors to mid-exponential phase at 30 to 45°C. The analysis revealed that HrcA played a central regulatory role in PHSR while CtsR was not detected in any fraction. The RNA polymerase subunits α, β and δ were detected in CFEs and at the cell surface as highly up-regulated after culture at supra-optimal temperatures and sigma factor σ^A (σ^{70}) was moderately up-regulated in the CFEs, while sub-units β′, ω and presumptive ε were down-

regulated at 45°C, suggesting modulation of RNA polymerase was involved in PHSR. YycF ortholog inhibitors, considered as attractive bactericidal agents against pathogenic bacteria, could impact the ability of *L. casei* GCRL163 to cope with growth at elevated temperature, as response regulator YycF_2 was induced at 45°C. Orthologs of proteins under the regulation of σ^B in *Bacillus subtilis* were up-regulated at 45°C, including Asp23-domain proteins Asp23_2, YqhY and BN194_17970. Several proteins associated with transcription, translation and post-translational modification were differentially modulated by thermal stress. This study shows the importance of proteomics in investigating stress response regulation, which can complement the current transcriptomic-based knowledge in lactobacilli, particularly in identifying novel condition-specific regulators.

Metabolic processes, such as proteolysis, carbohydrate and amino acid metabolism, are pivotal in food biotechnology. Understanding the metabolic mechanisms underpinning the adaptation of the strain to prolonged heat stress is vital for improving bacterial functionalities. To investigate the modification of the metabolic pathways under prolonged heat stress, we further analysed the LFQ proteomic datasets of CFEs, cell surface-associated (TS and LS extracts) and ECF protein fractions of *L. casei* GCRL163, cultured to mid-exponential growth phase at 30°C, 40°C and 45°C and pH 6.5 in bioreactor systems. The proteomic data revealed upregulation of EIIBGal, Lev- and Man-family proteins, involved in PTS-uptake of sugars other than glucose, suggesting utilization of alternative sources of carbon. Proteins GalE_2, GalT, BGAL17, Cap4C and BN194_07390, involved in galactose and fructose metabolism were repressed. Networks of metabolic pathways were activated to channel carbon into the glycolytic pathway, including phospholipid metabolism coupled to up-regulated TpiA, pentose phosphate pathway shunt via upregulation of GntK and Gnd, tagatose metabolism by over-expressed PfkA, LacC, LacD2 and LacD2_2 and amino and

nucleotide sugar metabolism by upregulation of NagA, ManD and NagB. Proteins involved in nucleotide metabolism, peptidoglycan biosynthesis and high energy-requiring fatty acid biosynthesis were repressed at 45°C, while proteins associated with RNA degradation increased. This study demonstrates that a key mechanism of PHSR in *Lactobacillus* spp. involves efficient management of energy generation and utilization, particularly around carbon scavenging pathways.

In general, the results in this thesis provide an in-depth insight into understanding the mechanisms underlining PHSR in *L. casei* GCRL163 and the modulation of proteins involved in metabolic pathways and regulatory proteins that mediate PHSR in *L. casei* GCRL163. This thesis study has further shed more light on the impact of environmental stress conditions, including persistent heat and acid stress, on adhesion of *L. casei* GCRL163 to human cell lines, which can be important for probiotic strain selection.

LIST OF PUBLICATIONS

Paper published

- Kayode T. Adu, Anthony L. Baker, Richard R. Wilson, David S. Nichols, John P. Bowman and Margaret L. Britz. Proteomic analysis of *L. casei* GCRL163 cell-free extracts reveals a SecB homolog and other biomarkers of prolonged heat stress. *PloS one* 13, no. 10 (2018): e0206317**.**

Conference abstracts

- Kayode Adu, Anthony Baker, Richard Wilson, John Bowman and Margaret Britz (2017). Improved Surface Protein Recovery and Surface Proteome in *L. casei* GCRL163 under Prolonged heat stress. *ASM 2017 (Australian Society for Microbiology, Planetary Health Conference, 2017)*; Hobart, Tasmania, Australia; 2-5 July, 2017. (Poster presentation).

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- Kayode Adu, Anthony Baker, Richard Wilson, John Bowman and Margaret Britz (2017). Proteomics of *L. casei* GCRL163 under prolonged heat stress: proteome and sub-proteome. Biocenter 1, Department of Biosciences, University of Helsinki, Finland, Europe; 16 June, 2017. (Invited talk).

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LIST OF KEY ABBREVIATIONS

µg	Microgram
µL	Microlitre
µM	Micromolar
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GIT	Gastrointestinal tract
HPLC	High-performance liquid chromatography
kDa	Kilodaltons
L	Litre
M	Molar
m/z	Mass/Charge ratio
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MRS	de Man, Rogosa and Sharpe Medium
MW	Molecular Weight
nanoLC-MS/MS	Nanoscale liquid chromatography coupled to tandem mass spectrometry
NCBI	National Centre Biotechnology Information
OD	Optical Density
rpm	Revolutions per minute

RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
v/v	Volume per volume

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 General introduction

Lactic acid bacteria (LAB) are large group of bacteria recognized to confer health-boosting benefits on the host and the probiotic attributes have gained much attention (Ljungh & Wadstrom 2006). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) defined probiotics as "*live microorganisms which, when administrated in adequate amounts, confers a health benefit on the host*" (Food, Organization & Organization 2006). The FAO/WHO definition was, however, reworded to allow for more precise use of the term 'probiotic' by an expert panel convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2013 as "*live microorganisms that, when administrated in adequate amounts, confers a health benefit on the host*" (Hill *et al*. 2014). The LAB can act as bio-therapeutic agents to modulate immunity (Borchers *et al*. 2009), lower cholesterol (Kumar *et al*. 2012; Ooi & Liong 2010), prevent cancer (De Leblanc *et al*. 2007; Kumar *et al*. 2010; Mohammadi 2013), treat rheumatoid arthritis (de los Angeles *et al*. 2011; Hatakka *et al*. 2003) and improve lactose intolerance (Reid 1999; Suarez *et. al* 1995; Vonk *et al*. 2012). Members of the genus *Lactobacillus* have been considered a minor member of the human colonic microbiota with the proportions correlating with disease and chronic conditions (Heeney *et al*. 2018). Although, there have been cases of infection-site isolation of some probiotic bacteria of the genera *Lactobacillus, Leuconostoc, Pediococcus, Enterococcus* and *Bifidobacterium* especially in immunocompromised patients (Liong 2008), LAB are rarely associated with GIT infections and have been granted GRAS (generally regarded as safe) status (Tuohy *et al*. 2003). The LAB have also been extensively used in the industrial production of fermented food products, facilitating the development of organoleptic and hygienic quality of fermented products, such as fermented milk and cheese products, in dairy industries (Mäyrä-Mäkinen & Bigret 1998).

During the manufacture of fermented food products and passage through the GIT, LAB are exposed to several potential stressors, such as high or low temperature, starvation, low pH, bile salts, and changing redox and osmotic conditions (Ferrando *et al*. 2015; Papadimitriou *et al*. 2016; Rossi *et al*. 2016). Of these, heat stress is one of the most characterized and commonly encountered conditions experienced by various LAB (Di Cagno *et al*. 2006). The most studied heat stress response involves a rapid upshift in temperature whereby bacterial populations are exposed briefly to heat shock (Broadbent *et al*. 1997; De Angelis *et al*. 2004; Tripathy *et al*. 2016). Exposing LAB to a temperature up-shift triggers heat shock response (HSR) and has led to the identification of various heat shock proteins, including the molecular chaperones and proteases (Richter *et al.* 2010). However, several other stress responses that can foster better understanding of metabolic and physiological mechanisms, associated with cell survival and probiotic functions, can be induced during prolonged heat stress. Cells can be grown in sub-lethal heat conditions over a period of time without a change to the stress temperature and the period of exposure can be transient or prolonged (Papadimitriou *et al*. 2016). In the current work, prolonged heat stress was performed by growing *L. casei* GCRL163 in sub-lethal heat conditions over a prolonged period of time without a change to the stress temperatures.

In order to confer probiotic and other functional traits, LAB must adapt to and survive exposure to heat and other stressors during manufacturing processes, as well as the harsh conditions encountered in the food supply chain, and finally after being consumed. For instance, HSR, as one of the most important adaptive mechanisms for cell survival and normal functioning, involves the modulation of several general and specific stress proteins, especially the regulation of chaperone proteins and proteases associated with protein quality control systems (Gottesman *et al*. 1997; Sugimoto & Sonomoto 2008). The stress-responsive

proteins that comprise the HSR can include cytosolic proteins, proteins compartmentalised within the cell envelope, and proteins secreted into the growth medium (Silhavy *et al*. 2010). In prolonged heat stress, several of these proteins might be associated with key metabolic and physiologic processes which could overlap with other metabolic networks in cells. Collectively, these processes contribute to maximised cell survival and function to a greater level than what the HSR can achieve alone.

The expression of heat stress-responsive proteins is under the control of master regulatory proteins. The regulation of HSR has been extensively studied in model organisms such as Gram-negative *E. coli* (Bukau 1993) and Gram-positive *B. subtilis* at the transcriptome level (Schumann *et al*. 2002). The *in silico* genomic analysis of HSR regulation in *L. casei* and *L. rhamnosus* has also been reported (Rossi *et al*. 2016). However, little is known about the regulation of PHSR in bacteria, such as *L. casei,* at the proteome level. From the transcriptional point of view, HSR regulation involves two major mechanisms that regulate the expression of heat shock genes in *E. coli*, *B. subtilis* and *Streptomyces* spp and these include transcriptional repressors, such as the CtsR and HrcA system, and alternative sigma (σ) factors (Schumann 2016).

The cell envelope is the main structure affected by the heat stress by impacting on the membrane fluidity and functions through changes to the lipid membrane integrity (Russell *et al*. 1995; Teixeira *et al*. 1997). The chemical composition of the cell envelope is often altered during heat stress, as an adaptation to assist the cells to survive and specialized repair machineries are mobilized to maintain cell functions and integrity (Jordan *et al*. 2008). Several LAB are also known to interact and adhere to the host intestinal cells through the adhesive biomarkers associated with the cell envelope, thereby conferring potential benefits

(Saarela *et al*. 2000). Several adhesive biomarkers have been identified in LAB and they include lipoteichoic acids (den Camp *et al*. 1985), carbohydrates (Fanning *et al*. 2012), extracellular proteins (González-Rodríguez *et al*. 2012) and surface proteins (Zhu *et al*. 2016). Surface proteins may include integral membrane proteins, lipoproteins, cell wall-binding proteins, cell membrane-anchored proteins and anchorless surface proteins (Tjalsma *et al*. 2008). These surface proteins may interact with the host and mediate immunomodulation, chemical sensing and communication or cross-talk (Le Maréchal *et al.* 2015). Several surface proteins have been reported for their involvement in probiotic mechanisms, including glycolytic enzyme enolase, ATP synthase, acetyl CoA acetyltransferase, molecular chaperone DnaK, chaperonin GroES, OppA ABC transporter, glycosyl hydrolases, flavoproteins and 50S ribosomal protein L10 (Kainulainen & Korhonen 2014).

Identification of the surface proteins in bacteria with minimal or no contaminant from the cytosolic proteins is still a target to achieve in understanding how cell surface responds to stress. Occurrence of cytoplasmic proteins in surface protein extracts may be due to cell lysis during handling or autolysis during growth (Espino *et al*. 2014), moonlighting activity (Wang *et al*. 2013) and trapped proteins in membrane-bound vesicle structures (Olaya-Abril *et al*. 2014). Several strategies have been applied for the extraction of surface proteins in LAB, which could be grouped into chemical and enzymatic approaches. The use of lithium chloride as a chemical method for surface protein extraction has been utilised in several studies recently (Garrote *et al*. 2004; Greene & Klaenhammer 1994; Hussain *et al*. 1999; Lortal *et al*. 1992b; McGavin *et al*. 1993). However, the LiCl extraction method is considered by some researchers to be too harsh for surface protein extraction, leading to the leakage of contaminating cytoplasmic proteins (Johnson *et al*. 2016; Tiong *et al*. 2015).

Hussain *et al*. (1999) noted that LiCl extraction might not be the method of choice for the selective extraction of surface proteins from *Staphylococcus epidermidis* as LiCl induced DNA release with a decrease in cell viability. Indeed, Gram staining of *S. epidermidis* treated with LiCl revealed disintegration of >50% cells. Nezhad *et al*. (2010) noted contamination of LiCl extracts with cytoplasmic proteins while detecting upregulation of surface-located moonlighting proteins following culture of *L. casei* GCRL46 at low pH.

Enzymatic methods may involve shaving techniques which employ enzymes such as trypsin in the surface protein extraction. The shaving method was first used in *Streptococcus pyogenes* (Rodríguez-Ortega *et al*. 2006) and was later applied to Gram-positive bacteria such as *Bifidobacterium animalis* ssp. *lactis* (Zhu *et al*. 2016), *S. pneumoniae* (Olaya-Abril *et al*. 2013), *Lactococcus lactis* (Meyrand *et al*. 2013) and *Listeria monocytogenes* (Zhang *et al*. 2013), Gram-negative bacteria including *E. coli* (Walters & Mobley 2009), and also in eukaryotes such as *Saccharomyces cerevisiae* (Braconi *et al*. 2011) and *Schistosoma mansoni* (Castro-Borges *et al*. 2011). Contamination of surface proteins obtained by surface shaving has been attributed to bioinformatic misidentification of cellular localization, cell lysis and the concept of moonlighting proteins (Olaya-Abril *et al*. 2014; Solis & Cordwell 2011; Solis *et al*. 2010; Tjalsma *et al*. 2008). It is therefore expedient to assess membrane integrity following surface protein extraction (Solis *et al*. 2010). Plate counting method, although often used, is not a reliable method for bacterial cell viability and membrane integrity because some bacteria are viable but not cultivable (Hammes *et al*. 2010). Hence, more robust methods are required for proper assessment of the membrane integrity following surface protein extraction.

Quantitative proteomics has offered immense opportunities for the identification and quantification of various proteins involved in stress responses of LAB. Proteomics is described as large-scale, comprehensive and quantitative analysis of specific proteome or entire complement of proteins, including protein abundances, structures, functions, their modifications and variations as well as their interacting networks, for understanding cellular processes (Anderson & Anderson 1998; Blackstock & Weir 1999; Gorg *et al*. 2004; Martens *et al*. 2005; Wilkins *et al*. 2006). Proteomics may involve discovery-based analytical workflows (first generation proteomics) and quantitative methodology (second generation proteomics) (Champomier-Vergès *et al*. 2002). Several proteomic methods have emerged due to technological advancement, which can be grouped into global and targeted proteome profiling (Liebler and Zimmerman *et al*. 2013).

The general aim of the work reported in this thesis was to gain a better understanding of how LAB adapt to prolonged exposure to elevated temperatures, where PHSR may impact probiotic and starter culture functionality. The main approaches adopted involved the use of proteomic and bioinformatics tools, to identify and analyse protein fractions from *L. casei* GCRL163, containing largely cytoplasmic, surface and secreted proteins and their modulation in response to persistent heat when cultured under controlled growth conditions using bioreactor systems. This also involved determining the impact of prolonged heat stress on cell binding. The current chapter focuses on the review of LAB, their probiotic benefits and stresses experienced during industrial processes, storage and GIT passage. Furthermore, stress studies in LAB were reviewed with emphasis on PHSR and its regulation, cell envelope adhesive biomarkers and the involvement of proteomics in the identification and characterization of proteome. The results presented in Chapter 3 of this thesis describe proteomic analysis of *L. casei* GCRL163 CFEs, to reveal key proteins and metabolic

processes involved in adaptive strategies during heat stress. Surface proteins involved in PHSR in *L. casei* GCRL163 which could be associated with key metabolic strategies for adaptation and functions and how prolonged heat stress of *L. casei* GCRL163 impacted binding to human colorectal adenocarcinoma HT-29 cells are described in Chapter 4. The results reported in Chapter 5 decribe the comparative proteomic analysis of cell surface proteins from *L. casei* GCRL163 at mid-exponential and stationary phase, to reveal key metabolic and physiological changes, important for cell survival at the two growth phases. Proteins involved in the regulation of PHSR at the proteome level in *L. casei* GCRL163 are described in Chapter 6. The results presented in Chapter 7 describe changes in the expression of cytoplasmic, surface-associated and secreted proteins of *L. casei* GCRL163 that modulate metabolic pathways and signatures during prolonged heat stress.

1.2 Literature review

1.2.1 Lactic acid bacteria and lactobacilli

LAB is a term used to describe several genera of Gram-positive bacteria which ferment sugars to produce mainly lactic acid. The food-associated, industrially important genera include *Lactobacillus, Carnobacterium, Enterococcus, Oenococcus Leuconostoc, Pediococcus, Lactococcus, Tetragenococcus, Vagococcus, Weissella, Aerococcus, Abiotrophia* and *Streptococcus* (Fraqueza 2015; Klein *et al*. 1998; Makarova *et al*. 2006; Ng *et al*. 2015; Park *et al*. 2015; Scatassa *et al*. 2015; Sofu *et al*. 2015; Wang *et al*. 2015) and they are non-sporing, facultative or microaerophilic (see Stiles and Holzapfel 1997 for detailed reviews on taxonomy of food LAB). Members of the genus *Lactobacillus* are generally non-motile except for *L. agilis* and described *L. capillatus (*from stinky tofu brine) (Chao *et al*. 2008) and *L. ghanensis* (isolated from Ghanaian cocoa fermentations) (Nielsen *et*

al. 2007). Although *Lactobacillus* are ubiquitously distributed, forming part of the gut commensal microbiota shortly after birth, they are more abundant in carbohydrate-rich environments, such as the mucosa linings in humans and animals, plants, sewage, spoiling and fermenting food products (Bernardeau *et al*. 2008). They are typically classified as GRAS due to their safe use in food production, forming part of the human commensal microbiota in the GIT and constituents of fermented and non-fermented food products (FDA, 2010; Mojgani *et al*. 2015).

Among the LAB genera, *Lactobacillus* is one of the most studied as probiotics and currently consists of over 200 species (http://www.bacterio.cict.fr/l/lactobacillus.html) with guanine plus cytosine (G+C) content ranging from 33 to 55% (Savijoki *et al*. 2006; Stiles & Holzapfel 1997). There has been a continuous description of new species or reshuffling such that 55 species were described as of 1984, 103 as of 2003, 163 as of 2007 and 171 as of April 2015 (Coeuret *et al*. 2004; Garrity *et al*. 2004; Goldstein *et al*. 2015). These increasing numbers may be due to advancement in identification technologies particularly the use of sequence technology, as the use of conventional microbiological methods are not adequate to reliably identify strains to the genus or species level due to the similarities in their morphology, biochemical, and nutritional needs (Dubernet *et al*. 2002; Foschi *et al*. 2017). The genus *Lactobacillus* together with *Bifidobacterium* (a member of the phylum *Actinobacteria*) have recorded enormous importance in their industrial applications where they are used in dairy and biotechnology industries as starter cultures for dairy fermented food products, animal health and animal feed products (Li *et al*. 2014; Mojgani *et al*. 2015; Oakley *et al*. 2014; Park *et al*. 2015) at highly concentrated forms, of more than 10^{10} CFU/g (Coeuret *et al*. 2004). Ingestion of these bacteria in adequate amounts have been reported to alleviate a number of health challenging conditions in diabetes,

cardiac-related diseases, immune-deficient aliments and GIT-related diseases. Their beneficial roles may be related to their ability to survive the stressful conditions during probiotic formulations, passage through GIT and eventual adherence to the intestinal epithelia to elicit pathogen inhibition and exclusion (Etzold *et al*. 2014; Sengupta *et al*. 2013).

1.2.2 Probiotic health benefits of *Lactobacillus* species

The health benefits of most probiotics are achieved through their interactions with the host immune system, competition with pathogenic organisms in the GIT, mucosal barrier function improvement and production of anti-inflammatory and anti-microbial substances (Gogineni *et al*. 2013). Several *Lactobacillus* species were reported to improve hepatic conditions such as cirrhosis, hepatocellular carcinoma and viral hepatitis in humans by immunomodulation and reduction of pathogenic bacterial toxin among other mechanisms (Chávez-Tapia *et al*. 2015). Administration of *L. plantarum* NCU116 for five weeks to hyperlipidemic Sprague-Dawley rat model at 10^8-10^9 CFU/mL dosage was able to normalise the lipid metabolism and improve morphological structures of impaired hepatic and adipose tissues (Li *et al*. 2014). Furthermore, neutrophil dysfunction in patients with alcoholic cirrhosis is ameliorated after the ingestion of *L. casei* Shirota three times daily for 4 weeks at 6.5×10^9 CFU/mL dosage (Stadlbauer *et al*. 2008). The restoration of the neutrophilic phagocytic capacity might be due to reduction and subsequent normalized expression of Toll-like receptor (TLR) 4 and Interleukin (IL)-10 (Stadlbauer *et al*. 2008). The prevention and treatment of hepatic encephalopathy and improvement of liver damage in non-alcoholic steatohepatitis have also been associated with appropriate use of probiotics (Soriano *et al*. 2012). In chronic liver disease, probiotics improve the plasma level of malondialdehyde and 4-hydroxynonenal in both alcoholic and non-alcoholic liver cirrhosis patients. Administration of a mixture containing 450 billion bacteria of various strains (*S. thermophilus, B. breve,*

B. longum, B. infantis, L. acidophilus, L. plantarum, L. casei, and *L. bulgaricus*) for 120 days, generally improved routine liver damage tests and plasma S-nitrosothiols (Loguercio *et al.* 2005). Diarrhoea in infants with rotavirus enteritis and diarrhoea due to miscellaneous causes (enterotoxigenic *E. coli*, *Campylobacter*, norovirus), have symptoms ameliorated with the consumption of fermented products containing *L. rhamnosus* GG, *L. reuteri* ASM20016 and *L. casei* NIZO B255 (Marteau *et al*. 2001). These strains are capable of binding to the C-type lectin DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) with subsequent blockage of bystander T-cells by induction of Treg cells. This process is also involved in the treatment of inflammatory diseases, such as Crohn's disease and atopic dermatitis (Smits *et al*. 2005).

The effectiveness of probiotics in reducing high morbidity associated with Crohn's disease and ulcerative colitis, which are major forms of inflammatory bowel disease (IBD) seen in humans, has been reported (Reiff & Kelly 2010). Immunomodulatory activities of probiotics and induction of intestinal immune homeostasis are the major factors thought to contribute to the ameliorative mechanisms of probiotics. There is now evidence that probiotics may only be effective in mild to moderate cases with more research needed to determine their therapeutic effectiveness in more severe conditions (Reiff & Kelly 2010). There are more indications that the activities of probiotics in IBD, which can be associated with the development of colorectal cancer, may be due to pathogen exclusion by *L. rhamnosus* GG and Lc705 (Espino *et al*. 2015). It is suggested that soluble products secreted or produced in supernatant fluids by some probiotic bacteria, such as phospholipids, bacteriocins and proteins, may be effective for the treatment of IBD, as they reduce the incidence of sepsis that can be associated with bacterial administration (Mikov *et al*. 2014; Prisciandaro *et al*. 2009).

1.2.3 Industrial applications of *Lactobacillus* species in dairy industry

Several *Lactobacillus* species have been isolated from various fermented dairy products, such as yoghurt, vegetable and fruit juices, meat, cheese (Zhou *et al*. 2015) and fermented milk (Liu *et al*. 2015; Liu *et al*. 2015; Saraniya & Jeevaratnam 2015; Sosa-Castaneda *et al*. 2015) . Their presence is either due to deliberate addition during processing as starters or may form part of the indigenous microbial community or desirable "wild" contaminants in the fermented food (Bernardeau *et al*. 2008). They may be available as food and dietary supplements and marketed in some cases as pharmaceutical formulations in capsules, tablets and powders (Arora & Baldi 2015). Recently, there has been a major challenge to the industrial applications in some regions of the world especially in Europe and Australia where inexorable regulations may cause a drop in usage and marketing as most benefit claims are seen as mere propaganda without scientific authentication (Reid 2015).

In cheese, a solid-matrix fermented dairy product with high probiotic delivery potential to the gut (Ross *et al*. 2002), two major groups of bacteria are involved in the making and ripening which include those carefully selected and added as starters (starter lactic acid bacteria - SLAB) and nonstarter lactic acid bacteria (NSLAB) (El Soda *et al*. 2000). SLAB can be either primary or secondary starters. Providing an acidic condition through consistent lactic acid production during cheese making and the breaking down of protein and fat during cheese ripening are some of the major responsibilities of primary starters while the secondary starters are added for specific desired purposes such as the surface coloration (*Brevibacterium linens, Penicillium roqueforti* and *P. camemberti*) and gas production (*Propionibacterium shermanii* subsp. *freudenreichii* in Swiss cheese). They also participate in cheese ripening (El Soda *et al*. 2000). The most commonly encountered

Lactobacillus species in cheese ripening are the mesophilic (*L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*) and thermophilic (*L. helveticus*) groups. The NSLAB contribute to the flavour development and several *Lactobacillus* species, such as *L. paracasei*, *L. plantarum*, *L. brevis* and *L. casei*, are predominantly involved in high numbers (10^9 CFU/g) while pediococci and enterococci may be present in small quantities (Fox *et al*. 1998). The NSLAB are the dominant microbiota in cheese during ripening due to their high tolerance for stressful condition and therefore play a major role in the final quality and functionality of cheese (Settanni & Moschetti 2010). For instance, a bacteriocin-like inhibitory substance (BLIS) produced by *L. paracasei* NdP78 exerts an antimicrobial activity against *L. monocytogenes* and extended the shelf-life of fresh cheese Tosela with improved final sensory characteristics of the cheese (Settanni *et al*. 2011). Greater growth of coliforms and staphylococci were observed in the control without the strain of *L. paracasei* in the Tosela cheese than the factory-scaled cheese with the NSLAB, suggesting the efficacy of the probiotic antimicrobial activities.

1.2.4 Stressful life of lactic acid bacteria during industrial applications and GIT passage

Highlighting the industrial production of typical fermented dairy products is necessary for understanding various stresses LAB encounter during industrial processing of the fermented food products. The fermented and non-fermented acidophilus milk products are manufactured by addition of concentrated *L. acidophilus* to heat-treated milk, up to 140°C, to ensure microbiological stability of the final product during storage and fermentation in the fermented variance (Dunne 2001) (Figure 1. 1). The cultures, usually of human origin, are grown to high densities, concentrated and then dried before adding to the dairy products that serve as delivery carriers (Dunne 2001).

In fermented milk, heat treatment is also important for pasteurization of the milk and competitive survival of the probiotics during fermentation as they compete with possible contaminants (Robinson 1995). This procedure usually exposes the indigenous *Lactobacillus* species to thermal stress and the ensuing cooling and subsequent storage at 5°C also results in cold stress the probiotics must survive to remain viable. In the manufacture of yoghurt, which involves thermophilic protosymbiotic cultures of *S. thermophilus* and *L. deLrueckii* subsp. *bulgaricus,* fermentation is carried out at 40-45°C for 2.5-6 h after pasteurization and this is followed by cooling to 4-8°C (Heller 2001). The yoghurt medium is maintained at pH <4.8 by lactic acid produced by the *Lactobacillus* species and this is important for the stable gel formation from coagulated milk protein (Heller 2001). Manufacturing processes of cheese entail some critical steps, some of which introduce stress to the probiotics involved. The inoculation of pasteurized skimmed milk with mesophilic (e.g. *Lactococcus* species) or thermophilic (e.g. *L. helveticus*) starter cultures at about 33°C, scalding at ≤57°C for 1h (38°C in Cheddar) of the curd-whey mixture depending on the cheese variety, dry-salting and eventual ripening at 10-15°C for ≥1 year are some of the stress conditions encountered (McSweeney *et al*. 1994).

In most dairy products, probiotics are added in the form of a dried powder and are exposed to very high temperatures in the spray-drying and also very low temperatures during freeze drying and storage, which may threaten the viability of the bacteria (de Figueiredo *et al*. 2015). In freeze drying, the bacterial cells can be frozen at temperature as low as -96°C and then dried under high vacuum by sublimation (Carvalho *et al*. 2004; Chen *et al*. 2015; Santivarangkna *et al*. 2007), which may expose the bacterial cells to peroxidation of the membrane lipids (Brennan *et al*. 1986) and disruption of secondary structures of DNA and RNA (van de Guchte *et al*. 2002). During spray-drying, increased cell membrane

permeability, loss of cytosolic structures, reduced metabolic activities due to changes in cell wall, DNA and RNA structural damage, and transition of lipid constituents of the cell envelope from lamellar to gel phase are some of the stressful occurrences (Desmond *et al*. 2001; Lapsiri *et al*. 2013; Mills *et al*. 2011b).

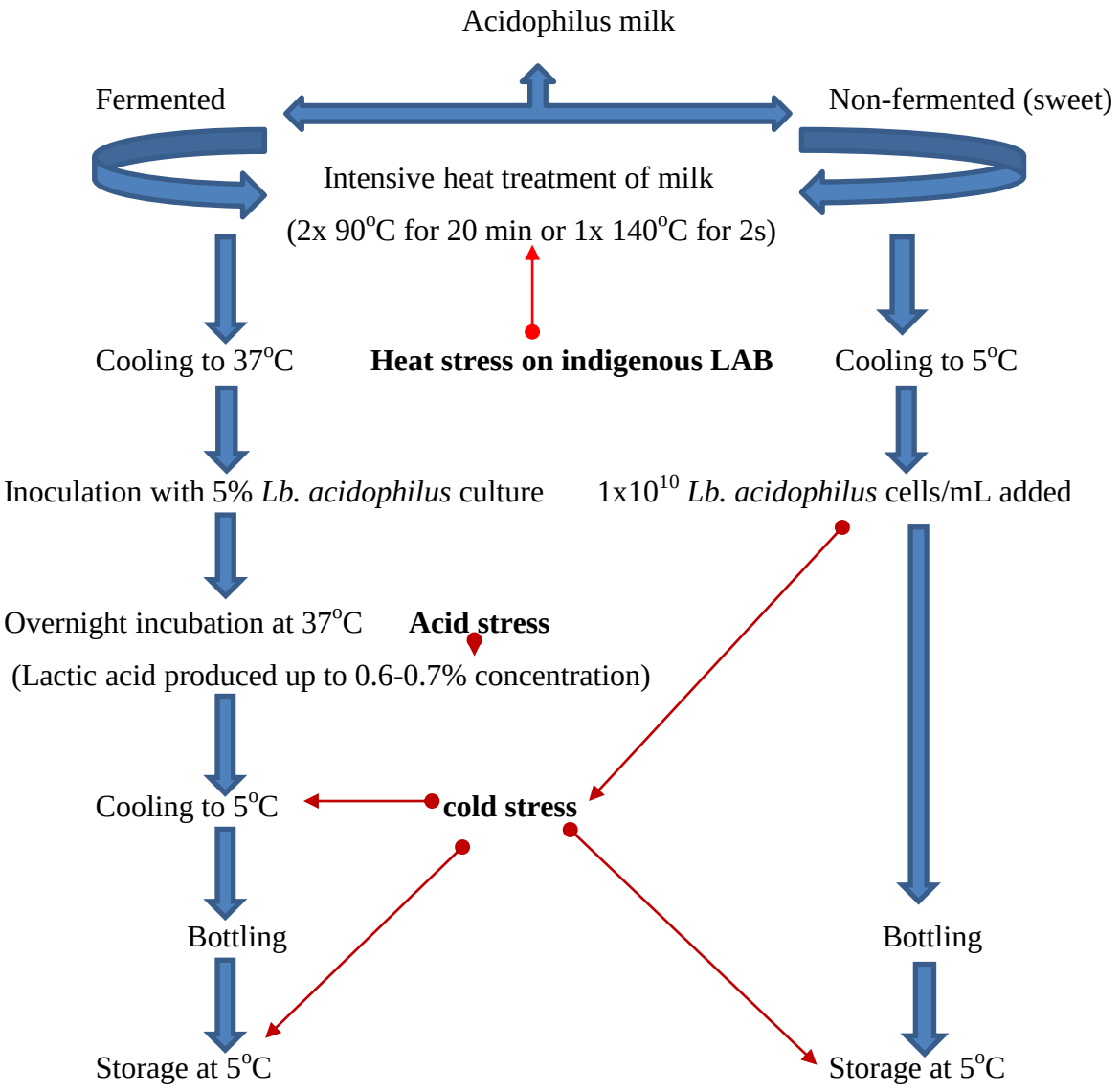


Figure 1. 1. Schematic diagram showing the production process of acidophilus milk with some of the possible stress conditions highlighted (heat, cold and acid) as adapted from (Heller 2001). Thermostable LAB are able to survive the intensive heat treatment.

The GIT, which includes the oral cavity through to the anal cavity, is a structure with the role of ingestion, digestion, absorption, assimilation and eventual evacuation of food and waste products (Kararli 1995). The GIT is also designed to greatly minimize survival of pathogenic organisms and in the process, other beneficial bacteria and probiotics are subjected to several stressful conditions (Berg 1996). The colonization of the human GIT with microbiota begins shortly after birth. LAB and bifidobacteria form parts of the complex microbial ecosystem in the GIT (van de Guchte *et al*. 2002; Vaughan *et al*. 2002). The intact intestinal epithelium acts as a barrier to the invasion of pathogens and any harmful antigens from the gut. The acidic pH of the stomach due to gastric acid (usually pH 1.5-3.5 and maintained by proton pump H^+/K^+ ATPase (Hoehn & Marieb 2010) inhibits the growth and survival of pathogenic organisms, the detergent-like bile secreted by the hepatic system for the emulsification of lipids and lipid soluble metabolism also exerts harsh conditions on pathogens, and the steep gradients of oxygen in the gut are some of the stresses faced by the gut microbiota, of which probiotics are unluckily included (Corcoran *et al*. 2008; Fuller 1991; Holzapfel *et al*. 1998).

Gastric acid and bile have been reported to disrupt the structure of cell envelope, DNA, RNA, protein folding and proton motive force (PMF), due to intracellular accumulation of protons with subsequent reduction in intracellular pH and transmembrane pH, leading to transmembrane transport dysfunction (Corcoran *et al*. 2008; van de Guchte *et al*. 2002; Vaughan *et al*. 2002). The survival of probiotic bacteria is traceable to a number of adaptive alterations in the cell structures and physiology. The acid tolerance response in most LAB is characterized by the adjustment in proton pumps, repair of macromolecules, production of alkaline molecules, cell membrane and metabolism alteration, induction of

transcriptional regulator pathways, changes in cell signalling and the upregulation of various proteins (Cotter & Hill 2003).

1.2.5 Type of bacterial stress

1.2.5.1 Heat stress

Heat, when high enough, may cause protein denaturation, aggregation, biochemical imbalance and membrane damage (van de Guchte *et al*. 2002). From extensive experimental reports by several researchers, heat stress in LAB could be classified into heat shock and prolonged heat stress (Broadbent *et al*. 1997; De Angelis *et al*. 2004). The heat shock stress is characterized by a rapid upshift in temperature accompanied by a period of exposure to thermal conditions (De Angelis *et al*. 2004). In prolonged heat stress, cells are grown in sub-lethal heat conditions over a period of time without a change to the stress temperature. Although the underlying physiologic mechanisms of the HSR and HAR may overlap, some degree of differences are expected, not only in the experimental set-up but also in the proteomic changes associated with the molecular and metabolic processes for cell survival and functions. This may be one of the factors that account for contradictory results often encountered in bacterial proteomic analysis (Lüders *et al*. 2009b). In LAB, the current knowledge of heat stress is based mostly on the HSR with few details available on PHSR.

Bacteria generally respond to rapid upshift change in temperature through multiple adaptive mechanisms to ensure cell survival and one of such mechanisms is the HSR characterized by upregulation of heat shock proteins encoded by heat shock genes (De Angelis *et al*. 2004). During heat shock stress, the folding of most proteins becomes chaperone-dependent with reduced spontaneous folding while transcription of DNA to RNA

and further translation are reduced (Narberhaus 2002) (Figure 1. 2). Heat shock proteins such as those specified in the *dnaKJ* operon, *groESL* operon, and small heat shock proteins (sHsps) are molecular chaperones involved in refolding of damaged cellular proteins and others such as Clp ATPases proteases, including ClpP and FtsH, degrade proteins that are beyond repair (Gottesman *et al*. 1997). Proteomic studies in heat-stressed *L. plantarum* WCFS1 and Δ*ctsR* mutant strains revealed the expression of genes encoding Clp ATPases and ClpP protease regulated by class three stress gene repressor (CtsR) regulon at translational level (Russo *et al*. 2012). N-terminal sequencing in *L. plantarum* heat shocked at 42°C for 1h in mid-exponential and stationary phase revealed upregulation of nine proteins including DnaK, GroEL, trigger factor, ribosomal proteins L1, L11, L31, S6 and DNA-binding protein II Hba and CspC (Straus *et al*. 1990). Exposing *L. paracasei* to elevated temperature of 52°C for 15 min led to abundance of proteins with GroEL as the major Hsp up-regulated (Desmond *et al*. 2004). Small heat shock proteins, which are chaperone-like proteins protecting cellular proteins from damage during stress, can be induced by other stresses, such as salt and acid stress and have been repressed by bile stress in *L. acidophilus* NCFM (Capozzi *et al*. 2011). The *dnaKJ* and *groESL* operons are controlled by negative mechanism involving the HrcA repressor protein binding to the cis acting element CIRCE (controlling inverted repeat of chaperone expression) in some LAB (Straus *et al*. 1990). Unlike in *B. subtilis* where CtsR and HrcA regulons are distinct from each other, they may overlap or one of them may be absent in some LAB, giving the regulation of the expression of both regulons to a single regulator (Elsholz *et al*. 2010).

The main target of heat is the plasma membrane due to its fatty acid composition. Heat stress on *L. bulgaricus* in skimmed milk at 64°C caused a cytoplasmic membrane damage with higher temperature resulting in cell wall and protein damage (Teixeira *et al*. 1997). Teixeira *et al.* (1997) also noted three major reversible peak responses in the cell

membrane lipids, ribosomes and DNA of the heat stressed bacterium with irreversible impairment occurring in the cell ribosomes when adapted in broth media at 65°C. A significant alteration in cell membrane integrity and esterase activities in *B. bifidum* was reported at 60°C heat shock but the effects can be improved by spray drying with protective agents such as gum Arabic, gelatin and pectin (Salar-Behzadi *et al*. 2013). Pre-treatment with heat shock has also been highlighted to improve viability after heat stress. Strains of *L. acidophilus* NCFM, *L. casei* LC 301 and *L. helveticus* LH212 are able to withstand 20 min high temperature by 27%, 5% and 11% respectively when pre-treated with heat shock at 54°C (*L. casei*) and 63°C (*L. acidophilus* and *L. helveticus*) (Broadbent *et al*. 1997). PCR amplified-*groEL* operon over-expressed in *Lactococcus lactis* (pGR01) and *L. paracasei* (pGR02) constructed using nisin-inducible expression system showed increased tolerance to other stresses such as osmotic high solvent stress (De Angelis *et al*. 2004). Co-cultures of *L. deLrueckii* subsp. *lactis* 193 and *B. longum* NCIMB 8809 did not only show improved thermotolerance but also exhibited enhanced expression of chaperone stress response compared to monocultures (Sánchez *et al*. 2013).

The regulation of the heat shock response involves two major mechanisms including transcriptional repressors and alternative sigma (σ) factors (Schumann 2016). The transcriptional repressors include HrcA and CtsR, which negatively regulate key heat shock genes including the *groESL* and *dnaKJ* operons and the genes encoding the Clp protease complex. The class I heat shock genes (*groESL* and *dnaKJ*) are regulated by the transcriptional repressor HrcA through binding to the Controlling Inverted Repeat of Chaperone Expression (CIRCE) that acts as an operator (Schulz & Schumann 1996). In *B. subtilis*, the alternative σ factor B (σ^B) regulates the expression of class II heat shock genes which code for general stress response proteins (Price *et al*. 2001). The CtsR regulon is

constituted by the *clpC*, *clpP* and *clpE* operons which recognize a tandemly repeated heptad operator sequence thereby serving as class III heat shock gene repressor (Derré *et al*. 1999). Other heat shock genes, such as *htpG* in *B. subtilis*, are under the control of a currently unknown regulatory system (Versteeg *et al*. 2003).

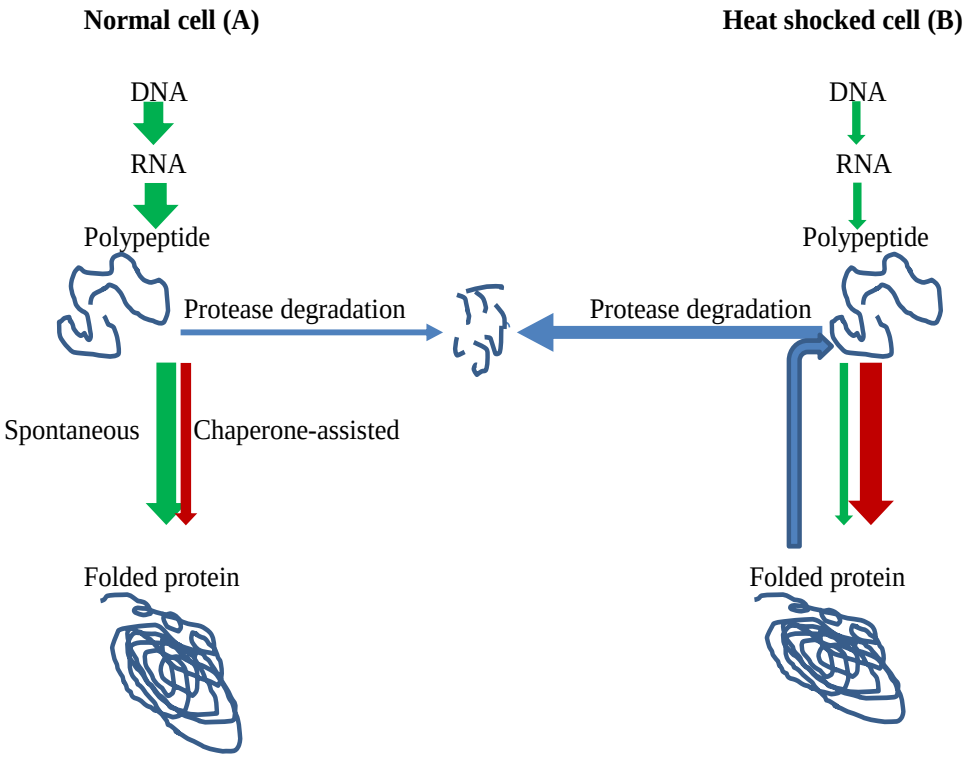


Figure 1. 2. Schematic illustration of protein quality control system under normal and heat shock conditions. (A) The rate of transcription and translation is very high in growth conditions without heat treatment and most proteins fold spontaneously without the assistance of chaperones (green thick arrow) with few aggregated and degraded proteins by proteases. (B) After heat shock, the transcription and translation capacity is reduced (green thin arrow) and previously folded proteins returned to the chaperone-dependent quality control system with most of the folding becoming chaperone-dependent (red thick arrow) (Narberhaus 2002).

In addition to HrcA and CtsR, two other negative repressors have been identified in Gram-positive *Streptomyces albus* including the heat shock protein repressor (HspR) and

repressor of *hsp*18 (RheA) expression (Grandvalet *et al*. 1997; Servant *et al*. 2000). The HspR binds to HspR-associated inverted repeat (HAIR) and represses expression of the *dnaKJ* operon while RheA binds to an inverted-repeat sequence to repress *hsp18* (Grandvalet *et al*. 1997; Servant *et al*. 2000). Further, most bacteria code a housekeeping σ factor, which regulates a large proportion of genes necessary for bacterial growth. However, bacterial genomes may contain several additional alternative σ factors, e.g. 3 in *L. lactis* (Bolotin *et al*. 2001) and 18 in *B. subtlis* (Kunst *et al*. 1997), which are induced under particular stress conditions (Kazmierczak *et al*. 2005). Although the involvement of alternative sigma factors in heat stress response has not been experimentally demonstrated, alternative σ^{32} and σ^{24} have been identified as positive regulators of HSR in *E.coli* (Schumann 2016) and in *L. casei* in *in silico* studies (Rossi *et al*. 2016). Several genes predicted as members of the σ^{32} regulons are involved in cellular adaptive mechanisms such as cell structure maintenance, translation and transcription systems necessary for nascent peptide maturations, transporters and carbohydrate metabolism (Kormelink *et al*. 2012; Rossi *et al*. 2016). An alternative sigma (H)-like factor in non-sporulating *L. sakei* was experimentally demonstrated to be hardly affected by growth at the stationary phase, demonstrating limited involvement in starvation response (Schmid *et al*. 2012).

1.2.5.2 Acid stress

Selecting potential probiotic bacteria that will still be viable to health benefits requires consideration of acidic nature of fermented dairy products as well as the gastric environment. Lactobacilli are known to generate lactic acid as fermentation end-products which upon accumulation create an unconducive environment for the growth of other bacteria. Lactic acid is known to have pKa of 3.86 and, in its undissociated form, is capable of diffusing through the cell membrane into the bacterial cytoplasm by a carrier-mediated

electroneutral process (De Angelis *et al*. 2004). Once in the cytoplasm, the lactic acid dissociates, constituting a growth-limiting factor to the cells in the presence of other stresses. Lactobacilli can tolerate an acidic cytoplasm up to pH 4.4 with extracellular media pH of 3.5. Below this level, cellular functions become impaired. Fermentation end-products, such as organic acids and alcohols, are responsible for the acidity and once these are accumulated, they affect the membrane lipids and integrity and act as protonophores to facilitate more inward flow of H^+ such that cytoplasmic basicity is also impaired (Kashket 1987). Other components of bacteria are affected as a result of the acid stress, including cell wall peptidoglycan, proteins and DNA. Cultivating *L. casei* at an acidic pH 4.0 leads to changes in the abundance of proteins on the cell surface compared to controls cultured at pH 6.5. Some fermentation and glycolytic enzymes such as lactate dehydrogenase, enolase and Gap become more abundant (Nezhad *et al*. 2012).

Several mechanisms have been identified in maintaining cytoplasmic pH homeostasis, providing cellular viability. The most important for fermentative lactic acid bacteria is the F_0F_1-ATPase complex (or H^+-ATPase) (Hutkins & Nannen 1993). The F_0F_1-ATPase complex can reverse its normal function and carry out H^+ extrusion by ATP hydrolysis (Al-Awqati 1986). H^+-ATPase was characterized in *Lactococcus lactis* subsp. *cremoris* HP to possess optimal activity at pH 5.0 as well as 7.5. In general, H^+-ATPase activity increases with a decrease in cytoplasmic pH until an extracellular pH of 5.0 when the activity then reduces (Nannen & Hutkins 1991). There is a higher acid tolerance associated with increased activity of H^+-ATPase in *L. casei* ATCC 4646 (Bender & Marquis 1987). Another acid-stress response mechanism in *Lactobacillus* is the arginine deiminase (ADI) pathway with three reactions catalysed by three enzymes, including arginine deiminase (ADI), ornithine transcarbamoylase (OTC), and carbamate kinase (CK) (Figure 1. 3). The pathway is

responsible for the conversion of arginine to ornithine, ammonia and CO_2 with one mole of ATP generated per mole of arginine used up (Zúñiga *et al*. 2002). The generation of ammonia (NH_3), carbon dioxide and ATP is driven by dephosphorylation of carbamoyl-phosphate. In *L. sanfranciscensis* CB1, ADI, CK and OTC were reported to have molecular masses of 46, 37 and 39 kDa respectively and the pIs were between 5.07-5.2 with the arginine catabolism dependent on the presence of a carbon source such as glucose and arginine (De Angelis *et al*. 2002). Rimaux *et al.* (2011) argued that the acid tolerance in acid stressed *L. sakei* through ADI pathway might be due to the provision of additional energy source in form of ATP at stationary phase. The ATP facilitates the export of cytoplasmic protons by F_0F_1-ATPase thereby reducing acidity (Burne & Marquis 2000; Sanders *et al*. 1999). Therefore, F_0F_1-ATPase is involved in pH homeostasis via ATP synthesis and proton extrusion in LAB (Baker-Austin & Dopson 2007). Accumulation of nearly 80 mM NH_3 was associated with degradation of arginine in *L. casei* and the pH rose to alkaline state of pH 8.0 (Wu *et al*. 2012). The upregulation of F_0F_1-ATP synthetase gene expression in *L. rhamnosus* GG under pH 4.8 and pH 5.8 was accompanied by a lowered nucleotide and protein biosynthesis, suggesting the ability of the bacterium to modulate its pyruvate metabolism depending on the growth pH (Koponen *et al*. 2012). Acidic treatment of acid-adapted *L. plantarum* at pH 2.4, simulating low pH in the GIT, resulted in the absence of antibiotic resistance and up-regulated induction of histidine biosynthesis (*hisD*) (Šeme *et al*. 2014). Adapting *L. delbrueckii* subsp. *bulgaricus* to 30 min of acidic treatment (pH 4.75) resulted in increased tolerance to lower pH of 3.5 by 250-fold compared to the control and similar findings were reported in *L. collinoides* with 0.015% enhanced survival when adapted at pH 5.0 for 90 min (De Angelis *et al*. 2004). Other mechanisms for pH homeostasis in lactobacilli involve low pH-inducible DNA repair system and alteration to the cell envelope (Zhang *et al*. 2008),

malolactic fermentation, and urease and amino acid decarboxylation (Papadimitriou *et al*. 2016; du Toit *et al*. 2011).

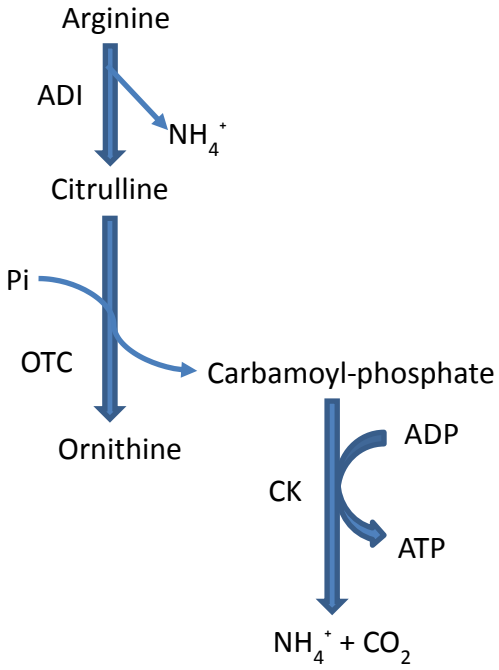


Figure 1. 3. The arginine deiminase pathway in *Lactobacillus sakei*. ADI-Arginine deiminase, OTC-Ornithine transcarbamoylase, CK- carbamate kinase (Zúñiga *et al*. 1998).

1.2.5.3 Starvation stress

One of the major growth-limiting challenges of probiotics in the GIT is the depletion in nutrients (Champomier-Vergès *et al*. 2002). Nutrient depletion facilitates the entrance of the log phase into the stationary phase as it does accumulation of fermentation end-products such as lactic acid (De Angelis *et al*. 2004; Heunis *et al*. 2014; Waddington *et al*. 2010). Classes of nutrient limitation identified in LAB include carbohydrate or lactose starvation, causing cell energy depletion, phosphate starvation, affecting the energy availability and DNA/RNA synthesis, nitrogen starvation, detrimental to the protein synthesis and other

inorganic nutrient starvation (De Angelis *et al*. 2004; Sengupta *et al*. 2013; Tong *et al*. 2017). Starved cells are exposed to such conditions as increased spontaneous mutation rate, chromosomal topology change, alteration in the plasma membrane fatty acid composition and cell wall structural change (Givskov *et al*. 1994; van de Guchte *et al*. 2002). The majority of changes observed in starved cells are thought to be survival responses to maintain cell viability and resistance to other stresses (Palumbo *et al*. 2004).

Several survival responses are activated in nutrient-depleted conditions in lactobacilli. Al-Naseri *et al*. (2013) reported the switch to scavenging mode of *L. casei* GCRL163 using Tween 80 and citrate spiced into the media containing different amounts of lactose in the range of 0%-1%. Expectedly, lactose and galactose catabolic pathway were inhibited under lactose limitation with upregulation of proteins involved in processes such as glycolysis, amino acid synthesis, pyruvate and citrate metabolic pathways (Al-Naseri *et al*. 2013). Earlier work on eight-day lactose-starved *L. casei* by Hussain *et al*. (2013) revealed upregulation of xylulose-5-phosphate phosphoketolase, elongation factor G and DnaK at stationary phase with nine proteins involved in protein synthesis, general stress responses and carbohydrate metabolism over-expressed as determined by two-dimensional electrophoresis (2-DE). Two general stress proteins, including the 60 kDa chaperonin and 20 kDa molecular chaperones, which were usually expressed in response to heat shock, were also expressed and detected during proteome mapping of *L. casei* GCRL163 subjected to lactose depletion (Hussain *et al*. 2009). After 75 days of starving *L. brevis*, metabolized arginine, glycine and histidine from dead *L. brevis* cells became the source of alternative nutrients and a number of enzymes involved in glucose, amino-acid and glycerolipid metabolizing pathways were up-regulated in response to stress as analysed by *de novo* sequencing in positive and negative mass spectrometry ion mode (Butorac *et al*. 2013). Yu *et al*. (2018) investigated *L. casei* Zhang

strains under glucose-limited conditions, using proteomic and next-generation sequencing analyses over a period of 3 years, and observed that proteins related to fructose and mannose metabolism, lyase activity and amino-acid-transporting ATPase activity were differentially induced. Expression of some proteins induced in response to starvation stress has been associated with response to other stresses like heat, cold, acid, oxidative or osmotic stress (Beaufils *et al*. 2007).

1.2.5.4 Osmotic stress

Lactobacilli are exposed to osmotic stress when higher levels of sugars or salts are added to the functional foods during processing and some are naturally subjected to osmotic imbalance due to their salty or sugary habitats (Piuri *et al*. 2005; Piuri *et al*. 2003). An alteration in the osmolality of the bacterial environment induces an adaptive response which may cause influx or efflux of fluids through the semi-permeable membrane of the cells (van de Guchte *et al*. 2002). Such response usually paves the way for accumulation of specific-low molecular weight compounds called compatible solutes or osmoprotectants in hyperosmotic conditions or their dissipation in hypoosmotic conditions through activation of transport systems. Compatible solutes include sugars such as trehalose and sucrose; amino acids such as glutamate and proline; polyols such as sorbitol and inositol and quaternary amines such as glycine betaine, and carnitine (Leslie *et al*. 1995; Silva *et al*. 2005). Glaasker *et al*. (1996) demonstrated that bacteria could respond to changes in the culture medium osmolarity by varying the specific compatible solute concentrations so as to maintain constant turgor pressure. The study reported increased cytoplasmic pools of K^+, proline, alanine, glutamate and glycine in *L. plantarum* ATCC 14917 when the osmolarity of the MRS growth media was increased from 0.20 to 1.51 Osmo/kg by KCl.

Most lactobacilli are generally known to have little or no capacity to synthesize compatible solutes and these solutes are necessary for enzyme stability, maintenance of cell membrane integrity during drying and enhancing positive turgor pressure thereby helping the cells to maintain osmotic balance with the environment (Bremer 2000). They may also protect cells against other stressors such as high temperature, drying and freeze-thawing (Kempf & Bremer 1998; Poolman & Glaasker 1998). Osmotic stress has been highlighted to affect cells at a structural level and in most cases, cells manifest physiological alterations. A strain of *L. casei* grown in high concentration of NaCl demonstrated larger cells than the control and further investigation, using transmission electron microscopy and analytical procedures, demonstrated a decreased peptidoglycan cross-link in cells with increased sensitivity to mutanolysin and antibiotics (Piuri *et al*. 2005). Hyperosmotic conditions studied in *L. casei* ATCC 393, in order to understand the influence of osmotic stress on the biochemical and biophysical characteristics of probiotic bacteria, revealed elevated hydrophobicity and bile salt sensitivity in the cells (Piuri *et al*. 2005). Glycolipid composition analysis by mono- and bi-dimensional thin-layer chromatography indicated an increase in tetrahexosyldiacyglycerol (H_4DG) level with a decrease in dihexosyldiacylglycerol (H_2DG) and *de novo* expression of acyltrihexosyldiacyglycerol (AcylH3DG) only in the stressed cells and not in the control (Machado *et al*. 2004).

1.2.5.5 Oxidative stress

Lactobacilli can be subjected to oxidative stress when there is a disruption in the oxidant balance in favour of pro-oxidants. Pro-oxidants are described as chemicals or substances that induce oxidative stress through the generation of reactive oxygen species such as superoxide anion (O^{2-}) and inhibition of antioxidant mechanisms (Puglia & Powell 1984). Oxidative stress has been described as an imbalance in the production of reactive oxygen,

reactive nitrogen species and antioxidant defence, resulting in various cellular pathophysiological conditions (Flora 2006). Cells under oxidative stress are liable to different cellular alterations including DNA mutations, strand breaks in DNA protein structure damage and oxidation of membrane phospholipids (Firuzi *et al*. 2011; Guerzoni *et al*. 2001). Lactobacilli being facultative anaerobic, do not require the presence of oxygen for growth, although they tolerate oxygen and their growth is better under anaerobic conditions. In fact, they are able to reduce pyruvate to lactate thereby resupplying NAD^+ to glycolysis.

Contrary to the general belief that lactobacilli cannot use oxygen as terminal electron acceptors, it is now clear that some lactobacilli possess NADH oxidase and can utilize heme for active respiratory chain system, although oxygen is associated with negative effects in LAB (Lechardeur *et al*. 2011; Rochat *et al*. 2006; Serrazanetti *et al*. 2013; van de Guchte *et al*. 2002; Vido *et al*. 2005). Respiring lactobacilli usually carry genes encoding electron donor NADH dehydrogenase and cytochrome *bd* oxidase, an electron acceptor (Brooijmans *et al*. 2009). In some lactobacilli, such as *L. bulgaricus*, the presence of oxygen can cause partially reduced toxic intermediates of oxygen to be generated such as superoxide anion, hydrogen peroxide and hydroxyl radicals. Lack of superoxide dismutase to eliminate superoxide anion in most lactobacilli in order to detoxify the cells can lead to eventual breakdown of anion to hydroxyl radicals with more accumulation of the radicals thereby causing cellular damage (Silva *et al*. 2005).

Presence of oxygen can also affect the fermentation output as cellular respiratory metabolism is altered and in the presence of heme, can lead to metabolic reprogramming thereby affect the tolerance of the cells to other variables such as temperature, pH and cell survival. Removal of such substances as hydrogen peroxide is one of the survival

mechanisms from oxidative stress in lactobacilli. Heterogeneous nonheme catalase produced by *L. casei* was demonstrated to be effective in removing H_2O_2 and *L. plantarum* ATCC 14431 degraded the deleterious compound using a nonheme, manganese-dependent catalase (Rochat *et al*. 2006). The expression of the catalase gene in *L. casei* did not only enhance oxidative resistance but also protect *L. bulgaricus* from oxidative stress of H_2O_2 by removing H_2O_2 produced by *L. bulgaricus* from the culture medium (Rochat *et al*. 2006). Similar to response in other stresses, pre-exposing lactobacilli to sub-lethal levels of H_2O_2 can protect them from cellular damage in subsequent exposure to lethal concentrations (Condon 1987).

1.2.6 Cell envelope: constituents and probiotic significance

The cell envelope of *Lactobacillus* species being Gram-positive is composed of a protein-embedded lipid bi-layeric cell membrane surrounded by a thick multilayered cell wall, which has been implicated in the conferral of probiotic properties (Sengupta *et al*. 2013). The rigid protective cell wall consists largely of peptidoglycan (PG) with associated teichoic acids including wall teichoic and lipoteichoic acids; pili, and covalently and non-covalently cell wall-anchored proteins (Sengupta *et al*. 2013). The PG is the predominant component of the bacterial cell wall constituted by glycan chains cross-linked with short peptide chains, forming a complex biopolymer which determines the shape and integrity of the bacteria (Chapot-Chartier 2014). The PG is elastic, and this helps the bacteria to overcome stretching forces due to bacterial turgor pressures, hence may form part of the bacterial survival mechanisms. The PG has a strand-form of architecture with selective permeability for some proteins and the threads of the strands are polymers of covalently linked alternating residues of N-acetyl-glucosamine (GlcNAc) and β-1-4-linked N-acetyl-muramic acid (MurNAc) held together by crosslinked pentapeptide side chains consisting of alternating L- and D- amino acids which attaches to the D-lactyl carboxyl group of MurNAc

(Chapot-Chartier 2014) (Figure 1. 4). The glycan strands and peptides contribute to strain diversity within and between species (Sengupta *et al*. 2013; Veiga *et al*. 2006).

The PG from *L. acidophilus* has been demonstrated to exert an anti-inflammatory action on LPS-induced inflammation in RAW 264.7 cells (Wu *et al*. 2015). Similar anti-inflammatory findings were observed in PG from *L. johnsonii* and *L. plantarum* with inhibition of *L. casei*-induced interleukin (IL)-12 and IL-12p40 expression through pattern recognition receptors such as toll-like receptors 2 (TLR-2) and nucleotide-binding oligomerization domain 2 (NOD2) but similar treatment failed to inhibit IL-12p35 mRNA (Shida *et al*. 2009).

Teichoic acids are chains of phosphodiester-bound glycerol or ribitol residues which are covalently linked to the PG through the C_6 of MurNAc residue as wall teichoic acids (WTA) or to the plasma membrane as lipoteichoic acid (LTA) by lipid anchors (Kleerebezem *et al*. 2010). The linkage unit of teichoic acids is structured by a disaccharide N-acetylmanosaminyl β (1-4) glucosamine followed by glycerol phosphate. The hydrophobic and anionic contributions of the teichoic acids to the cell wall have been reported and this enhances adhesiveness of the cells to the intestinal wall observed in most probiotic bacteria (Van Loosdrecht *et al*. 1987). Granato *et al*. (1999) reported the inhibitory interference of lipoteichoic acid in the adhesion of *L. acidophilus* La10 to Caco-2 intestinal epithelial cells at pH 4-7 and similar response was not observed in *L. johnsonii* La1 suggesting the involvement of LTA in probiotic adhesive properties of *L. johnsonii*. Lipoteichoic acid in *L. plantarum* exerts anti-inflammatory effects on human intestinal epithelial cells by inhibiting Pam2CSK4-induced interleukin (IL)-8 through their lipid and D-alanine moieties (Noh *et al*. 2015).

Cell wall polysaccharides in most lactobacilli can be covalently bound to the MurNAc of the PG, forming a thick outer layer called capsule and hence capsular polysaccharides (CPS) or loosely attached to the cell wall without forming a thick outer layer (wall polysaccharides - WPS) or in some species may form extracellular polysaccharides (EPS) in which they are not attached to the PG and can easily be released into the extracellular medium. Recently, Ibarburu *et al*. (2015) highlighted EPS, produced by two *L. suebicus* strains from Cider using Gas Chromatography/Mass Spectrometry, might consist of glucose, galactose, N-acetylglucosamine and phosphate. The findings by Ruas-Madiedo *et al*. (2006) revealed the involvement of EPS in the pathogen exclusion mechanism of some probiotics. Although the EPS of *L. rhamnosus* GG, *Bifidobacterium longum* NB667 and *B. animalis* IPLA-R1, failed to affect adherence of the probiotic bacteria to the intestinal wall, they influenced the adhesion of pathogenic bacteria such as *E. coli* NCTC 8603 and *Clostridium difficile* [recently renamed as *Clostridiodes difficile* (Lawson *et al*. 2016)] ATCC 9689 thereby reducing their virulence (Ruas-Madiedo *et al*. 2006).

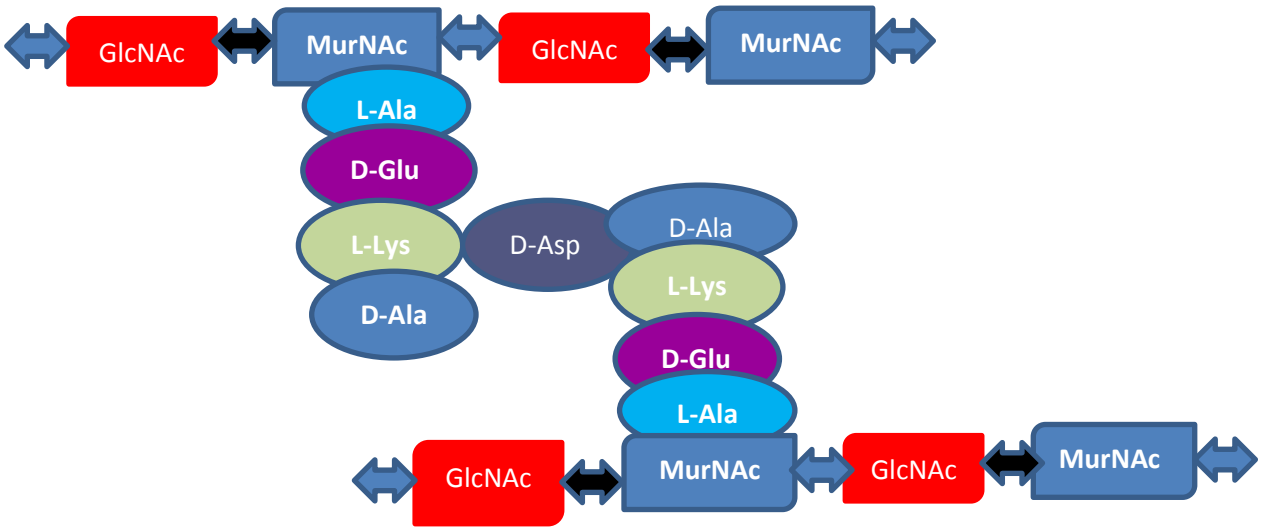


Figure 1. 4. Schematic representation of a typical lactobacilli peptidoglycan structure showing the arrangement of the constituents. Peptidoglycan is a polymer of glycan strands

formed by N-acetyl-glucosamine (GlcNAc) and β-1-4-linked N-acetyl-muramic acid (MurNAc) held together by crosslinking pentapeptide side chains of alternating L- and D-amino acids which attaches to the D-lactyl carboxyl group of MurNAc. L-Ala (L-alanine), D-Glu (D-glutamic acid), L-Lys (L-Lysine) and D-Ala (D-alanine) form the pentapeptide side chains (Chapot-Chartier & Kulakauskas 2014).

1.2.7 Cell surface proteins and moonlighting proteins

Cell surface proteins may anchor to the cell wall or secreted into the extracellular surroundings and become reassociated with the cell surface under certain physiological conditions such as change in acidity or belong to the group of proteins with no known export mechanisms (Espino *et al*. 2015; Kainulainen *et al*. 2012). Several lactobacilli produced surface (S)-layers, which have been described as crystalline arrays of self-assembling, lattice-like, proteinaceous subunits constituted by S-layer proteins which completely cover the cell surface (Hynönen & Palva 2013; Johnson *et al*. 2013b. Removal of S-layer proteins by lithium chloride resulted in low auto-aggregation and adhesion to HeLa cells compared to a control (Ventura *et al*. 2002). The S-layer proteins from *L. crispatus* ZJ001 could compete with *S. typhimurium* and *E. coli* 0157:H7 for binding to the HeLa cells resulting in competitive exclusion (Chen *et al*. 2007). The covalently anchored proteins are bound to the cell wall through various strands of attachment, which may be lipid-anchored (lipoprotein) and LPXTG-anchored with the N-terminally anchored proteins constituting the largest class of cell surface-anchored proteins in lactobacilli. Unlike C-terminally anchored proteins with no known function, the N-terminally anchored proteins are involved in cell envelope metabolism, signal transduction, extracellular transport and protein turnover in lactobacilli (Båth *et al*. 2005; Kleerebezem *et al*. 2010). The lipoproteins which constituted the second largest membrane-anchored proteins in lactobacilli are transported by the secretory (Sec) pathway and may be involved in adhesion, antibiotic resistance, folding and translocation of

proteins (Kleerebezem *et al*. 2010; Sengupta *et al*. 2013). The LPXTG-anchored proteins possess an N-terminal signal sequence with a type-I SPase cleavage site in its C region.

The existence of multifunctional proteins is beginning to complicate the interpretation of the functions of homologous proteins in bacteria as more proteins are being identified, using advanced proteomic technologies, to have other functions different from their primary roles. A protein can therefore be found as an enzyme in glycolysis and also play a significant role as adhesive factor in probiotics on cell surface (Jeffery 1999). Such proteins are described as moonlighting proteins. Jeffery Constance, who was believed to have coined the term "moonlighting proteins" in 1999, described in her reviews the exclusion of moonlighting protein from those that become multifunctional as a result of gene fusions, splice variants, protein isoforms, families of homologous proteins or a product of promiscuous enzyme activities (Jeffery *et al*. 2014; Jeffery 2009; Jeffery 2014). However, the concept of multifunctional proteins has been reported first in '80s by Joram Piatigorsky and Graeme Wistow while working on delta-crystallin proteins (Piatigorsky *et al*. 1988). They observed striking sequence similarity between structural protein delta-crystallin and the enzyme argininosuccinate lyase, which though encoded by the same gene, perform different functions (Piatigorsky *et al*. 1988). Following the advancement in protein identification techniques and the use of proteomics, over 300 moonlighting proteins have been reported (Amblee & Jeffery 2015; Jeffery *et al*. 2015). Many of these moonlighting proteins are cytosolic enzymes, molecular chaperones or other proteins with secondary function in other cellular location, other cells or as part of multi-protein complexes localised on bacterial cell surface (Jeffery *et al*. 2015).

Moonlighting proteins were previously described as "gene sharing" proteins but this description was seen as being ambiguous as gene sharing had also been used in association with horizontal gene transfer (Piatigorsky *et al*. 1988). They were also described as "anchorless proteins" or "non-classically secreted proteins" because their sequences possess no known sequence motifs for surface anchoring and no identified secretion signals (Pancholi & Chhatwal 2003). The multifunctionality of moonlighting proteins may involve two different functions at different cellular locations by a protein within a cell, as seen in *E. coli* PutA protein with proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase enzymatic activities when associated with cell membrane but in cytoplasm, it loses the enzymatic function to only act as transcriptional repressor by binding DNA (De Spicer & Maloy 1993). Furthermore, some proteins may have one function in the intracellular matrix while performing other functions extracellularly. Glyceraldehyde-3-phosphate dehydrogenase is a ubiquitous cytosolic protein, involved in the enzymatic phosphorylation of glyceraldehyde 3-phosphate to 1, 3-bisphosphoglycerate in the fifth step of glycolytic pathways. However, its moonlighting activities on cell surface have been demonstrated with at least three other functions including adhesion (Espino *et al*. 2015), anti-inflammation (Martín *et al*. 2015) and immunomodulation (Johnson *et al*. 2013a) in lactobacilli (Table 1. 1). In *L. helveticus,* moonlighting proteins are involved in adhesion to HT29 cells and also support the bacteria in colonizing the GIT through cellular aggregation (Wasko *et al*. 2014). Espino *et al*. (2015) reported the expression of 14 and 7 moonlighting proteins in *L. rhamnosus* Lc705 and GG respectively, which played significant role in immunostimulatory functions of the probiotic bacteria as analysed by 2-DE immunoblotting.

Several other proteins have been demonstrated to possess moonlighting ability, including stress response-related- cytosolic proteins belonging to the Clp family such as

ClpE, ClpX, ClpE, ClpB1/2, and ClpC, and translation-associated proteins (Espino *et al*. 2015). Glutamine synthetase and glucose-6-phosphate isomerase are moonlighting proteins released by *L. crispatus* STI following stress such as pH change or human antimicrobial peptide LL-37 produced during inflammation (Kainulainen *et al*. 2012). These proteins were able to bind effectively to the basement membrane preparation Matrigel made of type IV collagen and laminin, at acidic pH (Kainulainen *et al*. 2012). *L. acidophilus* NCFM was demonstrated to bind to Caco-2 intestinal epithelial cells *in vitro* by moonlighting proteins identified using LC-MS/MS analysis (Johnson *et al*. 2013a). Glycolytic enzyme enolase produced on cell surface of *L. plantarum* could mimic pathogenic organisms for binding sites on collagen to impair colonization and invasion of host tissues by the pathogens (Salzillo *et al*. 2015). Raghunathan *et al.* (2014) reported that enolase from *L. gasseri* inhibited binding of *Neisseria gonorrhoeae* to epithelial cells by competing for the binding sites.

Table 1. 1. Examples of moonlighting proteins produced by *Lactobacillus* species and their probiotic roles

Lactobacilli	Treatment	Identification	Moonlighting proteins	Roles	Reference
L. helveticus T159	48 h incubation at 42°C	Label-free analysis	Phosphoglycerate mutase, Enolase, Phosphoglycerate kinase, DnaK, GroEL, Elongation factor-Ts, Oligopeptide ABC transporter substrate binding protein, 30S ribosomal protein S1	Adhesion Aggregation	(Wasko *et al*. 2014)
L. rhamnosus	Overnight growth at 37°C	LC-MS/MS analysis	Gap, Chaperonin GroES, Endopeptidase O, Pyruvate dehydrogenase, Protein translocate subunit SecA, Phosphoglycerate kinase, Glucose-6-phosphate isomerase	Adhesion Immunostimulation Pathogen exclusion	(Espino *et al*. 2015)
L. crispatus ST1	16-18h growth at 37°C	SDS-PAGE, Blotting	Glucose-6-phosphate isomerase, Glutamine synthetase, Gap, Enolase	Adhesion	(Hurmalainen *et al*. 2007; Kainulainen *et al*. 2012)
L. acidophilus NCFM	16 h growth, 37°C	LC-MS/MS	Gap, RpsC-30S ribosomal protein, LysA-diaminopimelate decarboxylase	Adhesion Immunomodulation	(Johnson *et al*. 2013a)
L. casei BL23	Growth at 37°C	LC-ESI-MS/MS	Gap, Enolase, ClpL, GroEL, EF-Tu, EF-G	Adhesion	(Muñoz-Provencio *et al*. 2011)
L. plantarum LM3, LM-CC1	Growth at 30°C	2-DE	Enolase	Inhibition	(Salzillo *et al*. 2015)
L. plantarum 423			Triose phosphate isomerase, EF-Tu, Gap	Pathogen exclusion Adhesion	(Ramiah *et al*. 2008)
L. reuteri ZJ614-623	Growth at 37°C	MALDI-TOF-TOF	Gap	Adhesion	(Zhang *et al*. 2015)
L. jensenii	Growth at 37°C	2-DE	Gap	Adhesion	(Martín *et al*. 2015)

1.2.8 Factors affecting surface proteins expression and probiotic activities

Several factors have been described to influence the expression of proteins on the bacterial cell surface. Extracellular localisation of moonlighting proteins and binding to the cell surface are affected by acidity or alkalinity of the culture medium (Espino *et al*. 2015; Kainulainen *et al*. 2012). The medium pH altered the location of some surface-associated proteins, including glutamine synthetase, glucose-6-phosphate isomerase, enolase and Gap (Antikainen *et al*. 2007; Hurmalainen *et al*. 2007). In alkaline condition, they bind to the cell surface and at acidic pH, they are released into the medium, thereby constituting major parts

of the extracellular sub-proteome in most acidophilic lactobacilli (Hurmalainen *et al*. 2007). Moonlighting proteins are thought to be associated with the cell surface through ionic bonds as the model for attachment of enolase and Gap to the cell surface by negatively charged anchoring molecules has been reported (Kainulainen *et al*. 2012). It is now known that the proteins bind to lipoteichoic acids of the cell envelope at an acidic pH lower than their isoelectric point (IP) (Antikainen *et al*. 2007) and surface properties are modified secondary to pH changes (Antikainen *et al*. 2007; Kainulainen *et al*. 2012). The acidic environment of the GIT may therefore enhance host-bacterial interactions and adhesion through released proteins.

Capsule formation in some lactobacilli during stationary growth phase, as part of structural surviving mechanism, has been highlighted to have negative effect on the binding capacity of these bacteria. Cells at the exponential growth phase possess few capsules and have been observed to possess higher adhesive properties compared to cells at stationary growth phase. In *L. acidophilus* CRL 639, the presence of capsule formed at the stationary phase resulted in the inhibited adhesion to collagen-I and fibronectin (Lorca *et al*. 2002). Also, the preference of fibronectin binding to proteins of molecular weight of 15kDa and 45-58 kDa and proteins binding collagen-I, as analysed using monoclonal antibodies in *L. acidophilus* CRL 639, may indicate the influence of protein molecular mass in the adhesion of cellular proteins expressed in most lactobacilli (Lorca *et al*. 2002). Most moonlighting proteins are within the range of 200-600 amino acids, although some moonlighting proteins such as alcohol acetaldehyde dehydrogenase and pyruvate-ferredoxin oxidoreductase possess 866-870 and 1157 amino acids, respectively (Amblee & Jeffery 2015).

1.2.9 Proteomics in stress studies of *Lactobacillus* species

The separation and identification of proteins from complex mixtures using polyacrylamide gel electrophoresis in a one-dimensional technique became prominent for its useful applications in early biological research (Bell 1962). This technique, although useful, has faced several challenges especially in complex biological system where pleiotropic effectors, mutation and developmental transitions could not be properly analysed. This prompted the design of a high-resolution two-dimensional polyacrylamide gel electrophoresis by O'Farell in 1974. This new approach was based on the separation of proteins using two parameters which include isoelectric point by isoelectric focusing in the first dimension and molecular weight by sodium dodecyl sulfate electrophoresis in the second dimension (O'Farrell 1975). Although many researchers had previously worked on the strategies of two-dimensional electrophoresis as basis for ribosomal assembly, its output was not significantly better than one-dimensional electrophoretic analysis with limited resolution and applications (Held & Nomura 1973). With advancement in technology, there have been several improvements, with the concept of proteomics developed based on the principle of two-dimensional electrophoresis. The word proteomics was originally coined from "protein" and "genomics" in 1994 by Marc Wilkins and co-workers (Wilkins *et al*. 1996). The progress experienced in proteomics has been driven by the need for new technologies for peptide/protein separation, mass spectrometry analysis, isotope labelling for quantification and bioinformatics data analysis.

Proteomics can involve two approaches: bottom-up and top-down. Bottom-up workflows involve the extraction of proteins from cell samples using different procedures, depending on cell compartments followed by proteolytic digestion to peptides (Yates 2004). A bottom-up procedure performed on mixture of proteins is called shotgun proteomics which

provides an indirect measurement of proteins by analysing the peptides derived from proteolytic digestion of intact proteins (Yates 2004). Protein-protein interactions are destroyed in the process accompanied by loss of connectivity between the sites of post-transitional variants (Beynon *et al*. 2014; Rotilio *et al*. 2012). A typical bottom-up proteomic analysis involves the extraction of proteins from bacterial cells such as surface proteins (Lortal *et al*. 1992a). The protein sample is desalted or dialysed and peptide mixture fractionated (Zhang *et al*. 2013). In the top-down approach, intact proteins are injected into the mass spectrometer to avoid the challenge of interference associated with peptide-based proteomics (Catherman *et al*. 2014). However, this approach has significant limitations because of challenges associated with protein ionization, fractionation and fragmentation in the gas phase. A hybrid of the bottom-up and top-down approaches is being investigated as middle-down proteomics to minimize peptide redundancy between proteins (Moradian *et al*. 2014; Wu *et al*. 2012; Zhou *et al*. 2011).

Proteomic analysis has its own limitations, reproducibility of results and accurate identification of proteins can best be achieved under monitored stress conditions and advanced mass and database search analyses (Champomier-Vergès *et al*. 2002). According to Russo *et al*. (2012), proteomics should be a highly reproducible tool that could allow data from different studies to be compared. In stress studies such as prolonged heat stress, strict control of interference of other stressors such as high acidity, starvation and oxidative stress is critical for result reproducibility and data reliability. Moreover, limiting the number of stressors during evaluation of microbial stress is crucial for understanding key molecular and physiological changes underpinning stress responses associated with specific stress. When change in redox conditions of the culturing medium is not adequately monitored during a stress study, for instance, cells may be exposed to oxidative stress that can influence the

redox potential of the cells through reactive oxygen species (ROS) and other derivatives thereby altering responses of vital macromolecules and cell enzymatic reactions (Pedersen *et al*. 2012). Proteins involved in scavenging alternative sources of energy may be induced, with alteration to amino acid and fatty acid metabolism, if starvation stress is not strictly controlled (Al-Naseri *et al*. 2013; Papadimitriou *et al*. 2016). Also, auto-acidification through sugar fermentation during acid stress in LAB can result in the generation of lactic acid as fermentation end-product and significantly alter cell physiology (Even *et al*. 2002). If potential interfering responses are not guarded against or considered during experimentation, the comparability and interpretability of proteomic data may become arduous and doubtful. It is therefore crucial to conduct stress studies under strictly defined stress conditions as interference from other stressors can result in false positive or negative observations (Champomier-Vergès *et al*. 2002).

While several stress studies did not report or control interference of other stressors during experimentation, a number of experiments were performed to study stress in LAB under controlled stress conditions (Table 1. 2). In a transient multi-stressor adaptation study on *L. sanfranciscensis* CB1 involving pressure, heat, cold and osmotic stress conditions, exponential cells from buffered medium were exposed to adaptation conditions in fresh preconditioned media for one hour, as a measure to control the interference of auto-acidification. Another strategy applied was the use of buffered medium as reported in the study of changes in cell surface proteins in *L. casei* 46 under acid stress (Nezhad *et al*. 2012). In the nutritional stress study of *L. casei* GCRL163, using buffered mMRS medium to control auto-acidification, Al-Naseri *et al.* (2013) demonstrated that the strain could adjust its energy requirements during nutritional starvation and use citrate and Tween 80 when lactose or glucose was depleted. The application of fresh or buffered medium has limitations and may

not be as robust as the use of computer-controlled bioreactor systems, especially during mono-stressor study involving a specific stress in LAB. Koponen *et al*. (2012) investigated protein expression and phosphorylation in *L. rhamnosus* GG in acid stress under strictly controlled conditions using bioreactors and concluded that proteins, involved in central cellular pathways, exhibited phosphorylation which could be extensive in glycolytic enzymes. Acid shock experiment, to investigate physiological responses and cryotolerance of *L. deLrueckii* subsp. *bulgaricus* CFL1 exposed to acidification after fermentation, was performed under controlled bioreactor systems with pH maintained at 6.0 by computer-controlled addition of 6% NaOH, temperature at 42°C and agitation speed of 100 rpm (Streit *et al*. 2008).

The bioreactor system is a tank that permits several biological reactions to occur simultaneously in a liquid medium, using a computer-controlled system, thereby allowing well-defined growth conditions (Bastin 2013). The automation and standardization enabled by bioreactors in a controlled closed system could enhance understanding of specific stress conditions with little or no interference from unwanted environmental stress conditions (Martin *et al*. 2004). A typical benchtop bioreactor system has a temperature control system monitored by a resistance temperature detector (RTD) submerged in a thermowell (Shimizu 1993). Oxygen levels can be monitored using dissolved oxygen (dO_2) probe while the flow of air, carbon-dioxide and nitrogen is under strict control, to create for instance anaerobic conditions for LAB growth. It is also equipped with an agitation system for even distribution of nutrients, a feeding pump and effluent point. A submerged aerator allows for creation of aerobic conditions. The system is normally under computerized control (de Gooijer *et al*. 1996).

Table 1. 2. Stress responses in LAB highlighting how stress conditions are monitored.

LAB species	Type/nature of stress studied	Growth phase at stress	Other stressors controlled	Control and stress conditions	Protein/gene analysis	Some of the stress responses	Some of the key findings	Ref
L. paracasei subsp. paracasei F19	Prolonged shock stress. Stress introduced after cell wash with buffer. Multi-stressor: pH, temperature pressure, oxidative, osmotic, drying, starvation	Exponential	Not mentioned	**Control:** 37°C in modified MRS (Spicher broth) at pH 5.4 **Stress:** pH 4, 60 min pH 9, 60 min 15°C, 60 min 45°C, 60 min 1M NaCl 60 min RT, 60 min 350 MPa, 10 min, 60 min Glu10, 10% (w/w), 60 min	Genomic and quantitative proteomic analysis (LC-MS/MS)	Subcellular localization localization: Upregulation of cell wall and membrane proteins, downregulation of cytoplasmic proteins. SEED: carbohydrate metabolism, nucleoside and nucleotide biosynthesis, and membrane transport, REDUCTION: DNA metabolism and cell wall and capsule biosynthesis	Optimal preconditioning toward drying was predicted to be alkaline and high-pressure stress preconditioning	(Schott *et al*. 2017)
L. paracasei subsp paracasei F19	Prolonged Shock stress. Stress introduced after cell wash. Multi-stressor: pH, temperature oxidative osmotic, starvation	Mid-exponential	Not stated	**Control:** 37°C in modified MRS (Spicher broth) at pH 5.4 **Sublethal stress:** NaCl/KCl, 1M H_2O_2, 1.4mM, Acid, pH 4 Alkaline pH 9 Glu10, 7.6 mM Heat, 45°C Cold, 15°C	MALDI-TOF mass spectrometry, 2D GE coupled to ESI MS/MS	Osmotic stress-upregulation of CTP-synthase, alanine-phosphoribitol ligase, proline iminopeptidase	Implementation of MALDI-TOF MS protein profiling for the fast and comprehensive analysis of various stress responses is new to the field of bacterial stress responses	(Schott *et al*. 2016)
L. sanfranciscensis DSM 20451	Transient adaptation. Stress applied for 1 h in fresh preconditioned medium. multi-stressor: pressure,	exponential	Addition of fresh medium to prevent acid stress, Buffered medium	**Control:** 30°C **Stress:** Heat (43°C 30 min), cold (12.5°C for 1 h), osmotic stress (1.9% NaCl for 1 h), 80 MPa HHP at	Proteomics (MALDI-TOF mass spectrometry)	Only one slightly increased protein was specific to the HHP response and showed homology to a clp protease	At the proteome level, *L. sanfranciscensis* used overlapping subsets of stress-inducible proteins rather than stereotype responses. Specific pressure response does not exist in this	(Hörmann *et al*. 2006)

	heat, cold, osmotic			30°C, pH 3.7			bacterium.	
L. rhamnosus HN001	Transient heat and osmotic shock Multi-stressor: Heat and osmotic	exponential	Not stated	**Control:** 37°C **Stress:** Osmotic – resuspended in NaCl (0.0 – 0.7M). Heat-45°C – 60°C for 30 min in water bath	2-dimensional gel electrophoresis and N-terminal sequencing	The phosphocarrier protein HPr up-regulated in cultures after the log phase irrespective of the stress treatments used, GroEL was 10-fold up-regulated in exponential compared to stationary phase, 15-fold increase after heat shock in log phase and 1.5-fold in stationary phase	The stationary-phase-related factors include osmolyte synthesis and accumulation, onset of multiple stress resistance, and starvation-induced stress resistance.	(Prasad *et al*. 2003)
L. plantarum WCFS1 and Delta ctsR Mutant Strains	Transient heat shock. Mono-stressor: heat	exponential	Not stated	**Control:** Temperature, 30°C **Stress:** Temperature 42°C for 30 min	Proteomics (MALDI-TOF mass spectrometry),	Increase expression of chaperones GroES, GroEL and DnaK.	CtsR-mediated regulation of some members of the Clp family	(Russo *et al*. 2012)
L. plantarum DPC2739	Prolonged heat shock. Mono-stressor: heat	mid-exponential and stationary	Not stated	**Control:** 30°C, mid-exponential (5 h) Stationary (12 h) in MRS washed then 1h in sterile milk at 30°C. **Stress:** 30°C, mid-exponential (5 h) Stationary (12 h) in MRS washed then 1 h in sterile milk at 42°C, 45°C, 48°C.	2-dimensional gel electrophoresis and N-terminal sequencing and Western blotting	changes in the levels of expression of 31 and 18 proteins in mid-exponential- and stationary-phase cells	Heat resistance of *L. plantarum* is a complex process involving proteins in cell physiology, including chaperone activity, ribosome stability, temperature sensing, and control of ribosomal function	(De Angelis *et al*. 2004)
Lactococcus piscium Strain CNCM I-4031	Cold shock and cold adaptation. Stress applied 1h or mid-exponential. Mono-stressor: Cold shock, Cold adaptation	mid-exponential	Not stated	**Control:** 26°C **Stress:** 5°C, 1 h or to mid-exponential	Proteomics (Liquid chromatography (LC)-tandem mass spectrometry)	Upregulation of proteins involved in general and oxidative stress responses and fatty acid and energetic metabolism	2-DE profiles and up-regulated proteins were different under cold shock and cold adaptation conditions, suggesting a sequence of steps in cold adaptation; AdhE up-regulated 2-fold (cold shock) and 5.1 (cold adaptation), FabF up-regulated (cold shock) and	(Garnier *et al*. 2010)

							FabG up-regulated (cold adaptation) FFabH up-regulated in both.	
L. rhamnosus GG	Prolonged acid adaptation Mono-stressor: acid	stationary	strictly defined bioreactor conditions	**Control:** pH 5.8 **Stress:** pH 4.8	Proteomic (2-D DIGE), transcriptomic analyses (whole-genome DNA microarrays)	Up-regulated: F0F1-ATP synthase genes. Down-regulated proteins in nucleotide biosynthesis and protein synthesis	Proteins in central cellular pathways were phosphorylated, especially in glycolytic pathway	(Koponen *et al*. 2012)
L. casei strain 46	Prolonged acid adaptation Mono-stressor: acid	Late exponential	buffered MRS	**Control:** pH 6.5 **Stress:** pH 4.0	Proteomics (MALDI-TOF mass spectrometry)	Up-regulated glycolytic enzymes at cell surface (enolase, Gap, Ldh), GroEL,	Relative expression of surface proteins does not change if culture pH allowed to drift down.	(Nezhad *et al*. 2012)
L. sanfranciscensis CB1	Transient acid adaptation. Mono stressor: acid	Mid-exponential	Not stated	**Control:** pH 6.4 fresh medium for 1 h **Stress:** Acid- resuspended in pH 5.0 fresh medium for 1 h .	two-dimensional gel electrophoresis and N-terminal sequencing	Identified heat-shock proteins include DnaJ, DnaK, GroES and GrpE. Only GrpE showed an increased level of expression in the acid-adapted and acid-tolerant mutants as compared with non-adapted cells	N-terminal portion of YhaH showing the highest induction factor of those proteins specifically induced in the acid-adapted cells	(De Angelis *et al*. 2001)
Lactobacillus deLrueckii subsp. *bulgaricus* CFL1	Transient acid shock.	stationary	strictly defined bioreactor conditions	**Control:** pH 6 **Stress:** pH 5.25 for 30 min	Proteomics (MALDI-TOF mass spectrometry)	Proteins involved in energy metabolism, nucleotide and protein synthesis and stress response were modulated	Acid adaptation induced cross-protection.	(Streit *et al*. 2008)
Oenococcus oeni PSU-1	Transient ethanol shock. Mono-stressor: ethanol	Late-exponential	Not stated	**Control:** pH, 4.35 Temperature 28°C, 12% (v/v) H_20 **Stress:** pH, 4.35 Temperature 28°C, 12% (v/v) ethanol for 5 h	Proteomics (MALDI-TOF mass spectrometry), Transcriptomics (microarray)	Down-regulated: lactoylglutathione lyase and glucosamine 6-phosphate aminotransferase at proteomic and transcriptomic level. Reduced proteins related to cell envelope/wall biogenesis, chaperones, protein turnover	Functions related to protein synthesis are the main target of ethanol stress	(Olguín *et al*. 2015)

L. plantarum WCFS1	Transient and prolonged ethanol adaptation. Stress applied over 10 and 30 min; 24 h. Mono-stressor: ethanol	Transient-exponential Prolonged-stationary	Not stated	**Control:** 20°C, H_20 8% v/v **Stress:** 20°C, ethanol 8% v/v	transcriptomic analyses (whole-genome DNA microarrays)	**Transient** - Up-regulated genes involved in citrate metabolism and cell envelope architecture **Prolonged** - Increased citrate consumption, cell membrane composition modified	Induction of CtsR class III stress responses provides cross-protection against heat stress	(van Bokhorst-van de Veen *et al*. 2011)
L. casei	Lactose Starvation adaptation Mono-stressor: lactose starvation	stationary	Not stated	**Control:** 1% lactose at 30°C for 6 h, 12 h, 24 h, 48 h and 72 h **Stress:** 0% Lactose for 6 h, 12 h, 24 h, 48 h and 72 h	MALDI-TOF/TOF mass spectrometry	enzymes of various metabolic pathways involved in carbohydrate metabolism were differentially modulated, such as glycolytic pathway, to yield energy	Onset of stationary phase and starvation conditions confer multiple stress resistance in LAB	(Hussain *et al*. 2009)

1.3 Significance and objectives of the project

Heat stress is one of the most characterized and commonly encountered conditions experienced by various LAB. The most studied heat stress responses involve a rapid upshift in temperature whereby bacterial populations are exposed briefly to heat shock as a way of understanding heat shock response through which LAB are able to cope with extreme of heat. In practise, however, LAB populations are often exposed to prolonged periods of heating during fermented food processing which may elicit more complex network of metabolic and physiological processes than previously reported in the heat shock response, thereby facilitating better understanding of cell survival and functions. Advancement in proteomics has paved way for better understanding of intrinsic details associated with stress responses in LAB. Although proteomics has its own limitations as a first choice in the study of stress responses, reproducibility and reliability of results can best be achieved under monitored growth conditions. The study of prolonged heat stress in *L. casei* GCRL163 is therefore important to gain more insight into how LAB adapt to stress and overall, facilitating their exploitation in biotechnological and diary industries.

This project was specifically designed to:

- investigate the physiological and molecular mechanisms of *L. casei* GCRL163 to prolonged heat stress
- investigate prolonged heat stress response at the cell surface of *L. casei* GCRL163 and its physiologic impact on binding to HT-29 cell line.
- understand surface protein expression associated with prolonged heat stress response in *L. casei* GCRL163 at different growth phases
- understand the impact of prolonged heat stress on metabolic pathways and regulatory proteins of *L. casei* GCRL163.

CHAPTER 2

MATERIALS AND METHODS

2.1 General materials

2.1.1 Bacterial strains and cell lines

Lactobacillus casei GCRL163 was the main bacterial strain used in this project. The bacterial strain, *L. casei* GCRL163, was originally isolated from Cheddar cheese (Chandry *et al*. 2002) and speciation confirmed by multi-locus sequence typing (Al-Naseri *et al*. 2013) and whole genome sequencing (Nahar *et al*. 2017). Other *Lactobacillus* species, *L. helveticus* K1, *L. rhamnosus* NBRC and *L. paracasei* 7K07A2, were obtained from the Microbiology Research Laboratory culture collection, University of Tasmania, Australia. All strains were stored in 40% glycerol in MRS broth (Oxoid, Australia) at -80^{o}C. The human colon adenocarcinoma cell line HT-29 (Cell Bank, Australia), used in this project, was cryopreserved in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% foetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO), and stored in an ultra-low temperature of <-135°C under liquid nitrogen. Other bacterial strains used are described in the specific chapters, reporting their use, including storage and culture conditions.

2.1.2 Media preparations

2.1.2.1 De Man, Rogosa and Sharpe (MRS) agar and broth for bacterial culture

De Man, Rogosa and Sharpe (MRS) broth used in this study was obtained from Oxoid, Australia with the following constituents (g/L): 20 D-glucose, 10 peptone, 10 lab-

lemco powder, 5 yeast extract, 2 dipotassium hydrogen phosphate, 5 sodium acetate, 2 triammonium citrate and 0.2 manganese sulphate; and 1 m/L Tween 80. Preparation was formulated to the manufacturers' directions. Preparation of MRS agar involved the addition of 1% agar (Oxoid, Australia) to MRS broth. Autoclaving of media was at 121^{o}C for 15 min and fresh media was used in all experiments.

2.1.2.2 Dulbecco's modified Eagle's minimal essential medium (DMEM) for HT-29

Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma-Aldrich, Australia) was obtained from manufacturer as filter-sterilised liquid medium. Preparation of complete DMEM was performed by supplementing DMEM with 10% (v/v) FCS, 100 U/mL penicillin and 100 mg/mL streptomycin. Complete DMEM was routinely used for the culture of HT-29 cells unless otherwise stated.

2.1.3 Chemicals

All chemicals, reagents and solvents used were analytical reagents (AR) grade and supplied mostly by Sigma-Aldrich Chemical Company (Australia) and Bio-Rad Laboratories (CA, USA) except where another supplier is specified. Unless otherwise stated, all water used was distilled water (dH_20) prepared using a LABGLASS CASCADE purification system (Westlab Pty. Ltd, Australia) or deionised water prepared using Milli-Q_{plus} ultrapure water system (Millipore, Australia). All percentage figures are w/v unless otherwise specified.

2.1.3.1 Stock solutions

The following stock solutions and buffers were prepared as described below.

a) Acrylamide stock solution 40%.

b) Ammonium persulphate (10%): 0.05 g ammonium persulphate was dissolved in 0.5 mL of dH_2O.

c) Denaturation buffer: 42 g of 7M urea was added to 15.2 g of 2M thiourea and 360 mg of 30 mM Tris base in 50 mL of distilled water and the pH adjusted with HCl to 8.0. The volume was made up to 100 mL in dH_2O.

d) Developing solution: prepared by adding 6% sodium carbonate and 0.0004% sodium thiosulphate with 0.05% formalin (35% formaldehyde) added immediately before usage in 400 mL dH_2O.

e) Esterification reagent: methanol, chloroform and HCl were added at 10:1:1 (v/v).

f) Extraction solution: hexane and chloroform were added at 4:1 (v/v).

g) Fixing solution: prepared by mixing 50% methanol, 12% acetic acid and 0.05% formalin (35% formaldehyde) in 200 mL dH_2O.

h) Hydrochloric acid (1 M): hydrochloric acid (86.2 mL) was added to 800 mL of dH_2O. The stock solution was adjusted to a final volume of one litre and stored at room temperature.

i) Incubation or oxidizing solution: 0.02% sodium thiosulfate was prepared.

j) Internal working standard: prepared with 50 µg/mL methyl tricosanoate in chloroform

k) Phosphate buffered saline: 1L of PBS was prepared by dissolving 8g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 800 mL of distilled water. The pH was adjusted to 7.4 with HCl and distilled water added to a total volume of one litre.

l) Preserving solution: 30 mL glycerol was made up to 250 mL with dH_2O.

m) Silver solution (silver staining): 0.2% silver nitrate was mixed with 0.076% formalin (35% formaldehyde) immediately before usage in 200 mL dH_2O.

n) Sodium dodecyl sulfate (SDS) solution (10%): 10.0 g SDS was dissolved in 80 mL dH_2O then made up to 100 mL.

o) Stop solution: prepared by mixing 50% methanol and 12% acetic acid in 200 mL dH_2O.

p) Surrogate working standard: prepared with 500 µg/mL nonadecanoic acid in chloroform

q) Tris buffer pH 7.0, 40 mM: 4.84 g Tris-base was dissolved in 600 mL dH_2O and adjusted to pH 7.0 using 5 M HCl. The volume then was made up to one litre in dH_2O.

r) Tris-HCl buffer pH 8.0, 100 mM: 15.76 g of Tris-HCl was dissolved in 800 mL of dH_2O and the pH adjusted to 8.0 with 5 M NaOH. The volume then was made up to one litre in dH_2O.

s) Tris-HCl buffer pH 6.8, 1.25M: 37.8 g Tris-base dissolved in 150 mL dH_2O and adjusted to pH 6.8 with 5 M HCl then made up to 250 mL in dH_2O.

t) Washing solution: 35% ethanol in 200 mL dH_2O

2.2 General Methods

All media for *L. casei* GCRL163 were autoclaved using a Certoclave Multicontrol Benchtop Autoclave (CERTOclave sterilizer GmbH, Austria) for volumes less than 200 mL (121^{o}C for 15 min) and Tomy SX-700E Autoclave (Tomy Kogyo Co Ltd. Japan) for larger volumes of up to 800 mL in bioreactor/fermenter units. Centrifugation of cell cultures and protein extracts were performed at 15^{o}C and 4^{o}C respectively using an Eppendorf centrifuge (Eppendorf centrifuge 5417R) for volumes less than 1.5 mL and an Eppendorf centrifuge 5810R for volumes between 2 and 700 mL. Benchtop centrifugation was done using Sigma 1-13 Benchtop Centrifuge (B. Braun Biotech International, Germany). Ultra-centrifugation was performed using a Sorvall WX Ultra series (ThermoFisher Scientific, Australia). Incubation was done appropriately using Variable Temperature Incubator (Axyos Technologies Pty. Ltd. Australia), Dry Bath Incubator (Bio-strategy Pty. Ltd. Australia),

Shaking Water Bath (Ratek Instruments Pty. Ltd. Australia), New Brunswick Innova 44 Incubator Shaker series (Eppendorf, USA) and Terratec Gradient Shaker Incubator (Terratec Pty. Ltd. Australia). Stress studies were performed in Bioflo/Celligen 115 Benchtop bioreactor/fermenter systems (New Brunswick, an Eppendorf company, USA) that allowed computer-controlled growth conditions. Pouring of media was done in a laminar flow cabinet (Gelman Sciences Pty. Ltd. Australia) and chemical procedures performed in a Valentalert model HC00 fume hood (Conditionaire International Pty. Ltd. Australia). Culturing of the cell line HT-29 was performed in Safemate 1.2 Vision Bio-cabinets (Laftechnologies Pty. Ltd. Australia). Fine chemicals were weighed using a Sartorius TE1502S (Data Weighing Systems, USA) balance for large quantities and a Mettler Toledo analytical balance TLE models (VWR, Switzerland) for small quantities of chemicals. All pH measurements were performed using an Orion pH meter model 250A (ThermoFisher Scientific, Australia). Stirring and vortexing were done using magna-stir magnetic stirrer (Edwards Instrument Company, Pty. Ltd. Australia) and vortex mixers (Ratek Instruments Pty. Ltd. Australia) respectively. Optical densities were measured using a SPECTROstar spectrophotometer (BMG Labtech, Australia). Running of SDS-PAGE gels were done using the mini-Protean Tetra system (Bio-Rad, Australia).

2.2.1 Microbiological methods

2.2.1.1 Growth conditions and incubation of bacterial culture

The *Lactobacillus* strains used in this study was routinely plated on MRS agar and incubated anaerobically using an Anaerocult A system (Sigma-Aldrich, Australia) at 37°C for 48 h. Starter cultures were routinely prepared by inoculating a single colony into freshly prepared MRS (Oxoid) broth and incubating at 37^{o}C overnight before inoculating. 800 mL of

MRS (Oxoid) broth supplemented with additional 1% glucose in Bioflo/Celligen 115 Benchtop bioreactor/fermenter systems (New Brunswick, an Eppendorf company, USA) was routinely inoculated to a starting OD_{600} of 0.02 for stress experiments.

2.2.1.2 Growth conditions and culturing of the HT-29 cell line

Thawing of the HT-29 cells in cryovials was routinely done in a 37^{o}C water bath. Once thawed, the cryovials were surface-sanitised with 70% ethanol and placed in UV-sterilized cell culture hood. One millilitre of cell suspension was added to 10 mL of DMEM to dilute out DMSO. Cells were centrifuged at 300 × g for 5 min and resuspended in 12 mL of complete DMEM before transferring into a T75 flask (Sigma-Aldrich). The cell line was routinely cultured in a 37^{o}C incubator at 5% CO_2 until approximately 80% confluent. Subculturing of the cell line was performed whenever cell growth reached approximately 80% confluent or higher. Cells were washed once with pre-warmed sterile Earle's Balanced Salt Solution (EBSS) (ThermoFisher Scientific, Australia) and 2.5 mL of EBSS with 2.5 mL trypsin-EDTA added to detach cells. Cells were then collected by centrifuging at 3000 × g for 5 min and resuspended in 1 mL complete DMEM.

2.2.1.3 Preservation of bacterial cultures

Stock cultures of the bacterial strain used in this study were prepared by growing the strain on MRS agar plates for 48 h. Cells were harvested from agar plates using sterilized loops and suspended in 2 mL of glycerol storage broth with the components prepared separately, sterilized at 121ºC then mixed aseptically. Each vial was frozen immediately at -20ºC prior to transferring to -80ºC for long-term storage.

2.2.1.4 Preservation of HT-29 cell line

The HT-29 cells were gently suspended in 3 mL of DMEM containing 10% FCS and 10% DMSO in a T75 flask. One millilitre of the cell suspension was then pipetted into pre-labelled cryovials and the cryovials placed in Mr Frosty freezing container to achieve cooling rate of -1^{o}C/min in -80^{o}C freezer for short-term storage. Cryovials were then preserved in an ultra-low temperature of <-135°C under liquid nitrogen for long-term storage.

2.2.1.5 Method of biomass determination

Optical density (OD) values were used to determine the biomass of the strain. Samples were diluted in sterile MRS broth to keep the OD measurements in the linear range of the Beer-Lambert law (0.2 and 1.0) followed by measuring absorbance at 600 nm (OD_{600}).

2.2.1.6 Determination of growth patterns

A volume of the overnight culture at 37^{o}C was inoculated into 20 mL of MRS broth to give an initial OD_{600} reading of 0.02 and incubated at 25ºC, 30ºC, 35ºC, 37ºC, 40ºC, 45ºC and 55ºC using a Terratec Gradient Shaker Incubator for 24 h. Growth was monitored indirectly by measuring OD_{600} every 1 h for 24 h and these values were graphed against time using a semi-$\log_{10}$ scale for OD_{600}. The specific growth rate was determined using the function:

$$\mu = (\ln x_0 - \ln x_1)/t$$

where μ is the specific growth rate and x_0 and x_1 are changes in the OD_{600} at time *t*. Maximum specific growth rate μ_{max} was determined from the slope of μ and *t* (Georgieva *et al*. 2009).

2.2.2 Sample preparation for protein analysis

Unless otherwise stated, 100 mL of culture from fermenters set at 30°C, 35°C, 40°C and 45°C was harvested at mid-exponential growth phase by centrifuging at 9000 rcf for 10 min at 15°C. The cells were washed once with Tris-HCl buffer (0.04 M, pH 7.0) and then resuspended in an appropriate volume of Tris-HCl buffer (0.04 M, pH 7.0) to obtain an OD_{600} of 20 and stored at -20°C. Technical triplicates from each bioreactor unit were sampled for proteomic analysis.

2.2.2.1 Protein concentration determination

The Bio-Rad protein assay, based on the Bradford method (Kruger 2002), was used to determine the protein concentration using a bovine serum albumin (BSA) stock solution (2 mg/mL). A nanoDrop 800 spectrophotometer (ThermoFisher Scientific, Australia) at A_{280} was used for protein quantification of small volumes.

2.2.2.2 Protein analysis by gel electrophoresis

2.2.2.2.1 One dimensional SDS gel electrophoresis

One dimensional SDS gel electrophoresis was performed as described by Laemmli (Laemmli 1970) using 12% polyacrylamide gel in a mini-Protean Tetra system (Bio-Rad). Alternatively, a 4-12% TruPAGE precast gel, 10 x 8 cm, 12- and 17-well system (Sigma-Aldrich) was used with the TruPAGE LDS sample buffer (Sigma-Aldrich) following the manufacturer's instructions in the mini-protean Tetra system (Bio-Rad).

2.2.2.2.2 Sample preparation for one dimensional SDS gel electrophoresis

Protein samples for the SDS-polyacrylamide gels were prepared by mixing the protein samples with sample buffer at a ratio of 4:1 by a Ratek vortex mixer, heated for 10 min at

100^{o}C in Dry Bath Incubator (Bio-strategy Pty. Ltd. Australia). The samples were cooled on ice and spun at 22000 rcf (Eppendorf centrifuge 5417R) for 2 min to pellet any aggregate and the protein-rich supernatant was then transferred to a clean tube for loading onto gels. Precision$_{plus}$ protein All Blue standards (Bio-Rad) were used as molecular weight markers (protein ladder) and an appropriate amount of protein was loaded directly into wells.

2.2.2.2.3 SDS gel casting

Casting of mini-gels was routinely done by preparing 12% running gel solution (5 mL) and 5% stacking gel solution (2.5 mL) as follow:

12% **running gel solution (**5 mL) makes one gel.

- 1.65 mL distilled H_20 to 10 mL conical vial
- 2.0 mL 40% acrylamide solution was added
- 1.25 mL 1.5M Tris pH 8.8 added
-0.05 mL 10% SDS added

Then just before use, the following reagents were added:

- 50 µL 10% ammonium persulphate (APS).
- 3 µL tetramethylethylenediamine (TEMED).

5% **stacking gel solution (**2.5 mL) makes one gel.

- 1.7 mL distilled H_20 to another 10 mL conical vial
- 0.415 mL 40% acrylamide solution was added
- 0.315 mL 1.0 M Tris pH 6.8 mixed
-0.025 mL 10% SDS added

Then just before use, the following reagents were added

- 25 µL 10% APS
- 3 µl TEMED.

A vertical slab glass sandwich was then prepared by placing the short plate in front of the spacer plate and then properly fixed onto the casting frame and assembled into the casting stand. A 0.75 mm 12-well/17 well Teflon comb was inserted, and a mark made at about 7 cm below the comb (to determine the level of the running gel to be injected). The comb was then removed, and the leakage of the set-up tested with water. Filter paper was used to clean up the testing water by capillary. The running homologous gel mixture was then cast into the vertical slab glass sandwich up to the marked level and layered with isopropanol to make a smooth edge and remove air bubbles. Polymerisation was allowed for 20 min and the isopropanol removed completely. The stacking gel was added to fill up the rest of the plates to the brim with pipette and this was done carefully to prevent addition of air bubble. The comb was replaced, and the gel allowed to polymerize for 1 h.

2.2.2.2.4 SDS gel running

The plate with the gel was placed onto the cassette with the short plate facing inside. The cassette was then assembled in the buffer tank and inner chamber filled with the single-strength running electrode buffer to the brim. The comb was removed carefully and the protein sample, prepared as described above, was loaded in parallel with molecular weight markers. The outer chamber was filled up to the marked level. The cover was placed and then connected to power at red to red and black to black arrangement. Electrophoresis was performed at 100 V until the dye marker reached the bottom of the gel. The gel was then removed and subjected to staining.

2.2.2.2.5 Gel silver staining

The gel was routinely fixed in a fixing solution for 2 h or overnight and then washed in 35% ethanol for 20 min thrice. The gel was sensitized in sodium thiosulphate for 2 min. The gel was washed in dH_20 thrice for 5 min before silver staining for 30 min and then rinsing with dH_20 twice for 1 min. The gel was then placed in the developing solution until bands were visible. The solution was discarded, and band development stopped by adding stop solution for 5 min. Stop solution was washed off for 5min thrice and then preserving solution added for 20 min. All formaldehyde was added immediately before use.

2.2.2.3 Desalting and concentrating

Desalting of trypsin-digested peptide/protein samples was performed using reversed-phase zipTip$_{C18}$ tips (Millipore) according to manufacturer's instructions. Lithium chloride-containing samples were dialysed by using dialysis tubing (pore size molecular weight cut-off ≤1200, retention of compounds with a molecular weight >2000, Sigma, USA) against deionized water (4^{o}C). Concentration of samples were done using Amicon stirred cells with PLBC ultrafiltration discs, generated cellulose, 1000 NMWL, 63.5mm filter diameter (Merck PL, Australia).

2.3 Specific materials and methods

2.3.1 Material and methods used in Chapter 3

2.3.1.1 Bacterial strain and growth conditions

Lactobacillus casei GCRL163 (Al-Naseri *et al*. 2013; Chandry *et al*. 1998; Nahar *et al*. 2017), isolated from Cheddar cheese and stored in 40% glycerol/MRS

(Oxoid, Australia) broth in -80^{o}C at culture collection of University of Tasmania, was propagated on MRS agar and cultivated anaerobically using an Anaerocult A system (Sigma-Aldrich, Australia) at 37°C for 48 h. Prior to inoculation, starter cultures were prepared by inoculating a single colony into freshly prepared MRS (Oxoid) broth and incubating at 37^{o}C for 12 h.

2.3.1.2 Prolonged heat stress in the bioreactor systems

A set of four water-jacketed Benchtop bioreactor systems (New Brunswick, Eppendorf, USA) each with 800 mL of MRS broth were supplemented with 1% glucose. The temperatures were maintained at 30°C, 35°C, 40°C and 45°C and monitored by a resistance temperature detector (RTD) submerged in a thermowell containing glycerol. The media were inoculated to an optical density at 600 nm (OD_{600}) of 0.02 from overnight starter cultures (MRS broth, 12 h, 37°C). The bioreactors were maintained at an agitation of 50 rpm. Anaerobic conditions were maintained by sparging with nitrogen gas at 100% set-point using ring sparger at 0.5 SLPM gas flow while oxygen, carbon-dioxide and air were maintained at 0% set-point before inoculation and during growth. Oxygen level was monitored using dissolved oxygen (dO_2) probes and the pH set point maintained at 6.5 with automatic online addition of 2M NaOH. All set-points were programmed from BioFlo/CelliGen 115 control stations and the loops monitored using BioCommand with OPC computer software (Eppendorf, Australia). Three technical replicates were harvested from each bioreactor unit for preparation of protein fractions for proteomic analyses. Technical replicates were used rather than biological replicates as the laboratory did not have the number oaf bioreactor units required to run the number of biological replicates using the same inoculation source.

2.3.1.3 Cell lysis and protein extraction

Cultures were harvested at mid-exponential growth phase by centrifuging at 9000 rcf (Eppendorf centrifuge 5810R) for 10 min at 20°C and used for CFE preparation following the methods described by Nezhad *et al*. (2012). The cells were washed once with Tris-HCl buffer (0.04 M, pH 7.0) and then resuspended in an appropriate volume of Tris-HCl buffer (0.04 M, pH 7.0) to obtain an OD_{600} of~20 and stored at -20°C. Triplicate samples were taken from each bioreactor unit at different temperatures for the proteomic analysis. CFEs were prepared by mixing 0.5 mL of the cell suspension with 1 g Zircon beads (0.1 mm diameter; Daintree Scientific Pty Ltd, Australia) in 2 mL screw-capped plastic tubes and then subjected to ten bursts of 1 min followed by 1 min cooling on ice between bursts using TissueLyser II (Qiagen, Australia). Cell debris was removed by centrifugation at 14000 rcf for 30 min at 4C (Eppendorf centrifuge 5417R, Crown Scientific Pty Ltd, Australia) and supernatant fluid collected and stored at -80°C until analysis. Protein concentrations were determined using the Bradford protein assay (Kruger 1994).

2.3.1.4 Lipid extraction and fatty acid analysis

This was as previously described and applied with slight modifications (Dionisi *et al*. 1999). Briefly, 50 mg wet weight of the cells collected at mid-log, late log and stationary phases were washed twice with Tris-HCl buffer (0.04 M, pH 7.0) to remove residual MRS medium. Cells were resuspended in 3 mL of esterification reagent (10:1:1 [v/v] methanol: chloroform: HCl [Sigma-Aldrich, Australia]) followed by the addition of 100 µL of surrogate working standard (500 µg/mL nonadecanoic acid in chloroform [Sigma-Aldrich, Australia]), then the mixture was heated at 80°C for one hour. After cooling to room temperature, 1 mL of distilled water was added then the fatty acid methyl esters (FAMEs) extracted using 1 mL of extraction solution (4:1 [v/v] hexane: chloroform [Sigma-Aldrich, Australia]). The

emulsion was separated by centrifugation (Eppendorf centrifuge 5417R, Crown Scientific Pty Ltd, Australia) at 2000 rcf for 5 min and an aliquot (2 mL) of the upper phase was then collected and dried by evaporation under nitrogen at room temperature. The dried extract was dissolved in 1 mL of internal working standard (50 µg/mL methyl tricosanoate in chloroform [Sigma-Aldrich, Australia]). Only glassware was used throughout the procedures and the cells not treated with surrogate working standard were used as control. Fatty acid compositions were then resolved and identified using Gas Chromatography/Mass spectrometry (Dionisi *et al*. 1999).

2.3.1.5 Protein reduction/alkylation and in-solution trypsin digestion

Trypsin digestion was done following described procedures with slight modification (Wilson *et al*. 2010). Briefly, 90 µg of the protein samples were denatured by suspending in 100 µL denaturation buffer (7M urea, 2M thiourea and 30mM Tris pH 8.0) and then sequentially reduced and alkylated by incubating in 10 µL of 100 mM dithioreitol (overnight at 4^{o}C). The samples were treated with 10 µL of 500 mM iodoacetamide for 2 h in the dark at 25^{o}C and then co-precipitated by incubating overnight with 1 µg of trypsin (Sigma-Aldrich) in 1 mL chilled methanol at -20°C. The trypsin-methanol protein samples were then centrifuged for 5 min at 3000 rcf and precipitates dried and reconstituted in 100 µL ammonium bicarbonate (100 mM, pH 8.0) followed by trypsinisation for 5 h at 37^{o}C with 1 µg of trypsin added after 3 h to achieve the ideal protein: trypsin ratio (50:1). Digestion was terminated by freezing at -20°C.

2.3.1.6 Protein identification by nano-liquid chromatography and high- resolution tandem mass spectrometry (nanoLC-MS/MS)

The nanoLC-MS/MS analysis of the tryptic peptides was performed using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano HPLC system (ThermoFisher Scientific, USA). Tryptic peptides equivalent to 1 µg of each protein digest were loaded for 3 min onto a 20 mm x 75 µm trapping column packed with PepMap 100 3µm C_{18} resin at flow rate of 0.05 mL/min using a mobile phase composed of 98% water, 2% acetonitrile and 0.05% trifluoroacetic acid. Peptides were then separated at a flow rate of 0.3 µL/min on a 250 mm 75 µm analytical column packed with PepMap 100 2 µm C_{18} resin (ThermoFisher Scientific, USA) held at 40ºC. Multi-segment gradient from 97% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20% water) using three segments (3-10% B over 12 min; 10-40% B over 120 min; 40-50% B over 10 min), holding at 95% B for 20 min then re-equilibration in 3% B for 15 min was used. The LTQ-Orbitrap XL was controlled using XCalibur 2.2 (ThermoFisher Scientific, USA) and operated in data-dependent acquisition mode where survey scans (m/z 460-2000) were acquired in the Orbitrap at a resolving power of 60000. MS/MS spectra were concurrently acquired in the LTQ mass analyzer on the eight most intense ions from the FT survey scan. Unassigned and singly-charged precursor ions were not selected for fragmentation and 30-second dynamic exclusion (repeat count 1 exclusion list size 500) was used. Fragmentation conditions in the LTQ were: 35% normalized collision energy, activation q of 0.25, activation time of 30 min and minimum ion selection intensity of 3000 counts.

2.3.1.7 Database searching and criteria for protein identification

RAW files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 (http://www.maxquant.org/) using the extracted ion currents of matched peptides (Cox, Jurgen *et al*. 2011). The extracted MS/MS spectra were searched against the UniProt proteome for *L. casei* strain W56 (ID UP000003734) database comprising 3092 protein entries (www.uniprot.org) using the Andromeda search engine. The genome of *L. casei* GCRL163 has been shotgun sequenced (DDBJ/EMBL/GenBank accession number MODT00000000) but not closed (Nahar *et al*. 2017). Hence, *L. casei* W56 was used as the reference for *L. casei* GCRL163 proteins as the two strains are phylogenetically closely clustered on the basis of pfam analysis and the genome of strain W56 is closed. The MaxQuant default parameters for protein identification by LTQ-Orbitrap MS/MS and label-free quantitation (LFQ) were used, including a maximum of two missed trypsin cleavages, variable oxidation of methionine and fixed carbamidomethylation of cysteine. The false discovery rates (FDRs) for peptide-spectrum matches and protein identification were both set to 1%. MaxLFQ was used for peptide intensity determination and normalization, based on pair-wise comparison of unique and razor peptide intensities and a minimum ratio count of 2 (Cox *et al*. 2014).

2.3.1.8 Statistical analysis of identified proteins

The *ProteinGroups.txt* output file generated by MaxQuant was processed as follows: the normalised label-free quantification (LFQ) peptide intensity values, the numbers of razor and unique peptides for each of the identified proteins were imported into Perseus software version 1.5.031 (http://perseus-framework.org/). Protein groups identified as potential contaminants (prefixed with CON_) and proteins identified only by site or by reverse

database matching were removed and remaining observed intensity values were log_2-transformed. Proteins were filtered to exclude proteins detected in fewer than nine of the biological samples or on the basis of a single matching peptide. Missing values (reported as NAN and equivalent to zero LFQ intensity) were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances. To determine proteins that were significantly altered in abundance by heat treatments, a two-sided *t*-test using Benjamini Hochberg correction for multiple hypothesis testing was applied. A 1% FDR threshold was applied to determine statistical significance, unless indicated otherwise. A second dataset was generated by filtering to include proteins that were detected in a minimum of three replicates of any biological sample, to detect proteins only present at moderately high abundance at one or two specific growth temperatures. Identification of homologs to uncharacterized proteins was done through UniProt (Consortium 2016) and putative identity assigned where appropriate from protein sequence analyses by BLASTN through Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000) and the National Centre for Biotechnology Information (NCBI) (Coordinators 2017).

2.3.1.9 Bioinformatic analysis of identified proteins

Functional profiling of the identified proteins was done according to their GO category for molecular functions based on the functional annotations of the *L. casei* W56 (Hochwind *et al*. 2012). The *t*-value for each functional group was determined by T-profiler analysis using the function:

$$tP = \frac{\mu P - \mu P'}{s\sqrt{\frac{1}{Np} + \frac{1}{Np'}}}$$

Where $$s = \sqrt{\frac{(NP-1)\,x\,s^2P + (NP'-1)\,x\,S^2P'}{NP + NP' - 2}}$$

The *t*P is the t-value for a given protein group P; μP represents the mean expression log-ratio of the *NP* proteins in protein group P; μP′ is the mean expression log-ratio of the remaining *NP′* proteins and *s* is the pooled standard deviation obtained from the estimated variances for groups P and P′. The associated two-tailed *P*-value was calculated from the *t*-value using the *t*-distribution (Boorsma *et al*. 2005; Bowman *et al*. 2012; Kocharunchitt *et al*. 2012). The *t*-values were then imported into online bioinformatics resource, Gene Cluster (version 3.0) to determine functional annotation clustering using unsupervised hierarchical cluster analysis with complete linkage. Heat maps of the T-profiler data (*t*-values) and Z-scored LFQ protein expression data were generated using Java Treeview 1.1.6R4 and Genesis software, respectively. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al*. 2015) partner repository with the dataset identifier PXD007097.

2.3.1.10 Three (3)D-structural modelling of SecB proteins and phylogenetic analysis

Proteins annotated as the bacterial export chaperone SecB (IPR003708) or containing a domain related to the SecB-like superfamily (IPR035958) were identified in UniProt in *E. coli*, *Bacillus* and *Lactobacillus* species, including reviewed and uncharacterised proteins. FASTA sequences, and the sequence of an uncharacterised protein in the genome of *L. casei* GCRL163 with IPR035958 and DUF1149 domains, were submitted to Phyre2

(Kelley *et al*. 2015) for 3D-structural modelling, using intensive mode. Models were visualised and reviewed in Phyre2 using JSmol for secondary structures and confidence (normally >86% of residues modelled with >90% confidence) and in the UCSF Chimera package (Pettersen *et al*. 2004), with structural alignment made using the Matchmaker tool in the latter. The protein sequence of the SecB homolog of *L. casei* GCRL163 was submitted to BLASTP through KEGG to detect similar proteins in *Lactobacillus* species. FASTA sequences of these proteins, and others identified in UniProt as SecB homologs in *E. coli*, *Lactobacillus* and *Bacillus* species, were submitted for multi-(Clustal Omega) and pair-wise (Needle) sequence alignment (EMBL-EBI, www.ebi.ac.uk). Phylogenetic trees for identifying clades (EMBL-EBI, www.ebi.ac.uk) and hierarchical clustering (www.Phylogeny.fr) (Dereeper *et al*. 2010) were constructed. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al*. 2015) partner repository with the dataset identifier PXD007097.

2.3.2 Material and methods used in Chapter 4

2.3.2.1 Bacterial growth conditions

Lactobacillus casei GCRL163, *L. helveticus* K1, *L. rhamnosus* NBRC and *L. paracasei* 7K07A2, preserved in 40% glycerol/MRS (Oxoid, Australia) broth in -80°C at culture collection of University of Tasmania, were propagated on MRS agar and cultivated anaerobically using an Anaerocult A system (Sigma-Aldrich, Australia) at 37°C for 48 h. Strain identity was confirmed by sequencing PCR amplicons using universal primers for 16S rRNA genes and species-specific primers for *L. rhamnosus* (S. Shah, personal

communication). Starter cultures were prepared as described in section **2.3.1.1**, prior to inoculation

2.3.2.2 Prolonged heat stress in the bioreactor systems

Prolonged heat stress experiments were performed by maintaining a set of four water-jacketed Benchtop bioreactor systems (New Brunswick, Eppendorf, USA), each with 800 mL of MRS broth and supplemented with 1% glucose, at 30°C, 35°C, 40°C and 45°C and pH maintained at 6.5. Inoculation to an OD_{600} of 0.02 by overnight starter cultures (MRS broth, 12 h, 37°C) was done and anaerobic growth condition maintained as described in section **2.3.1.2.** Three technical replicates were harvested from each bioreactor unit for preparation of protein fractions for proteomic analyses.

2.3.2.3 Preparation of cell surface protein extracts by lithium chloride-sucrose

Extraction of the cell surface protein was done following the established method of Lortal and co-workers with minor modifications (Lortal *et al*. 1992b). Harvested cells from mid-exponential cultures (100 mL) were washed once using 20 mL Tris-HCl buffer (0.04 M, pH 6.8) and then re-suspended in 40% sucrose in 5M LiCl (0.1 w/v). Cell suspensions with no LiCl-sucrose added were used as controls. Cell suspensions were incubated at 4°C for 60 min and pelleted by centrifugation at 9000 rcf for 10 min at 4^{o}C (Eppendorf centrifuge 5810R). The supernatant fluid was filtered through 0.22 µm nitrocellulose membranes (Millipore, Australia) and surface protein extracts precipitated with -20°C chilled pure ethanol (1:9 v/v) by incubating for 2 h and centrifuging at 20000 rcf at 4°C for 30 min (Ma *et al*. 1996; Pepinsky 1991). Precipitate was then washed twice with 70% ethanol, to remove residual LiCl-sucrose, and resuspended in 300 µL Tris-HCl buffer (0.04 M, pH 6.8) for further proteomic analysis.

2.3.2.4 Preparation of cell surface protein extracts by trypsin shaving

Cell pellets, harvested from mid-exponential phase of 100 mL culture from the bioreactor units at 9000 rcf for 10 min at 15°C, were washed once with 20 mL Tris-HCl buffer (0.04M, pH 6.8). The pellets were then resuspended in 2 mL 0.05M Tris buffer containing 40% sucrose. Added as a reducing agent was 10 µL of 5M DDT followed by enzymatic digestion with 2 µg porcine trypsin/mg of cells (Sigma-Aldrich). Cell suspensions with no trypsin added were used as control. Incubation was done for 15 min at 37°C with 50 rpm agitation (Innova 44 Incubator shaker series; New Brunswick Scientific). Cells were pelleted at 9000 rcf for 10 min at 4°C and supernatant fluids containing proteins and peptides filtered using 0.22 µm nitrocellulose membranes (Millipore, Australia). The supernatant fluids were concentrated and desalted by filtering through zipTip$_{C18}$ with 0.6 µL C_{18} resin (Sigma-Aldrich) and additional 2 µg of trypsin added and incubated for 24 h at 37°C. The trypsin digestion was then stopped by storing in -20°C.

2.3.2.5 Preparation of extracellular fluid protein extracts by trichloroacetic acid-acetone

Cell culture fluid, from the cell suspension used for surface protein extracts, were concentrated to 2.5% of the original volume using Amicon stirred cells with PLBC ultrafiltration discs, generated cellulose, 3000 NMWL, 63.5mm filter diameter (Merck PL, Australia). The proteins were then precipitated by mixing 1 mL of the concentrate with 8 mL of 100% ice-cold acetone and 1 mL pure trichloroacetic acid (TCA) (1:8:1) for 45 min at -20°C. The acetone used was pre-chilled and stored in a -20°C freezer until needed and kept on ice during the entire procedure. The precipitated proteins were collected by centrifugation at 11500 rcf for 15 min at 4°C, soaked for 20 min in 100% ice-cold acetone at -20°C and washed thrice with 5 mL ice-cold acetone at 11500 rcf for 15 min at 4°C to remove residual

TCA. The final pellets were then dried at room temperature to remove residual acetone and then resuspended in 1 mL of Tris-HCl buffer (0.04 M, pH 7.4) and stored at -20°C to be used for further analysis. All protein concentrations were determined using the Bradford assay.

2.3.2.6 Determination of degree of cell lysis by DNA fragment analysis

Cell lysis was investigated by determining the concentration of released DNA fragments in the extracted cell surface protein extracts using Fragment Analyzer system (Advanced Analytical Technologies Inc. USA). 2 µL each from protein extracts obtained by trypsin shaving and LiCl-sucrose treatments were mixed with 22 µL of Genomic DNA Diluent Marker (DM) solution (Sigma-Aldrich) in a 96-well semi-skirted PCR plate of 12-capillary system (Eppendorf). 2 µL of the Genomic DNA Ladder were added to 22 µL of the DM in well 12 [Well H12 (96 capillary system)] and vortexing done at 3000 rcf for 2 min. The system was then run under the following conditions: perform rerun (6.0 kV, 30 sec), sample voltage injection (0.5 kV, 5 sec) and separation voltage (6.0 kV, 50.0 min). Extracts obtained from bacterial cells washed with distilled water were used as negative controls. Extracts from cells treated with ten bursts of one min followed by one min cooling on ice between bursts using TissueLyser II (Qiagen, Australia) were used as positive controls.

2.3.2.7 Determination of degree of cell lysis by flow cytometry analysis

The cell membrane integrity was investigated following the method described by (Salar-Behzadi *et al*. 2013). The cell samples (10 mL) were concentrated to OD_{600} of 1.0 (approximately 10^9 CFU/mL) and 10 µL of the samples was added to 985 µL PBS buffer. Staining was then performed by adding 5 µL propidinium iodide and incubated for 10 min at room temperature to allow complete labelling. Excess dye was washed off once by centrifugation at 9000 rcf for 10 min using 1 mL PBS buffer. The cells were then

resuspended in 250 µL of filter-sterilized PBS buffer (pore size, 0.2 µm). The working solution of the propidinium iodide used was prepared at a final concentration of 1.2 µg/mL. The florescence measurement was performed using a BD FACSCanto^{TM} II flow cytometer (BD Biosciences, Australia) at an excitation wavelength of 488 nm by using a 15-mW air-cooled argon laser at a sheath pressure of 12 lb/in^2 and florescence emission wavelength of 670 nm. The flow cytometry system was run such that 3000 events were collected for each sample at slow event rate to avoid detection of coincident events. Untreated stained cells were used as negative control and cells treated for 20 min at 70°C (heat-killed cells) were used as positive control. Change in mean fluorescent intensity (ΔMFI) was determined and dot plot analysis performed using Weasel flow cytometry software, version 3.2.1.

2.3.2.8 Determination of surface hydrophobicity of *L. casei* GCRL163

This was done according to the method previously described using a liquid-liquid two-phase partitioning assay (Microbial Adhesion to Hydrocarbons Test: MATH test) (Saini 2010). Cells from mid-exponential growth phase cultures of *L. casei* GCRL163 (OD_{600} approximately 0.8) were harvested by centrifugation, washed twice in 0.04 M Tris-HCl buffer, and resuspended in the same buffer. Bacterial suspensions (4 mL) were mixed with 1 mL of each solvent (chloroform, hexadecane and diethyl ether [Sigma-Aldrich]) in glass culture tubes and then vortexed for 2 min. The suspensions were allowed to separate for 15 min at room temperature and 1 mL of each aqueous phase was carefully withdrawn close to the base of the culture tube into glass cuvettes and OD_{600} measured. Three replicates were performed for each growth temperature and the control without cell suspension was performed. The percentage of cells binding to the solvents was then estimated using the equation:

$$\% \ MATH = 100 \ x \ [1- (A_1/A_0)]$$

where A_0 is the OD_{600} of the untreated control and A_1 is the OD_{600} of the treatments. The mean and standard deviations were determined using Statistical Package for the Social Sciences (SPSS) (Nie *et al*. 1970).

2.3.2.9 Binding assays of *Lactobacillus* strains to HT-29 cells

Adhesion of the four *Lactobacillus* strains (*L. helveticus* K1, *L. rhamnosus* NBRC, *L. casei* GCRL163 and *L. paracasei* 7K07A2) to human colon adenocarcinoma cell line HT-29 was assayed as previously described with slight modifications (Glenting *et al*. 2013). Briefly, HT-29 cell line was immobilized on eight-well chamber slides by culturing in Dulbecco's Modified Eagle's Minimal essential medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) foetal calf serum (FCS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in a 5% CO_2-humidified atmosphere for four days to reach 80% confluence and the cell culture media changed every 24 h. The bacterial cultures were centrifuged at 9000 rcf for 10 min at 20°C and washed once with Tris-HCl buffer (0.04 M, pH 7.0). Bacterial cells were re-suspended in sterile pre-warmed non-supplemented media (non-glutamine-DMEM/10% (v/v) without 1% P/S or 10% FCS) to adjust cell density to approximately 10^6, 10^9 and 10^{12} CFU/mL based on OD_{600}. Monolayers of HT-29 cells were washed twice with sterile Hank's balanced salt solution (HBSS) (ThermoFisher Scientific, USA) before the assays. For each adhesion assay, 100 µL of *Lactobacillus* strain suspensions were added to each well of the chamber slides containing HT-29 cells and incubated at 37°C for 1 h in 5% CO_2-95% atmospheric air. After incubation, non-adhered bacterial suspension was removed by washing five times with sterile HBSS and the cells fixed with methanol, Gram-stained, and examined under a microscope. For each monolayer, the number of

adherent bacteria was counted in 20 random areas and adhesion was expressed as the number of bacteria adhering to 100 cells of HT-29 cell line. When cells occurred in clumps or chains, each group was counted as one cell. Each adhesion assay was conducted in duplicate and the assays were repeated three times using HT-29 cells from three successive passages, with the results showing mean values of adhesive bacteria and the standard deviations estimated.

2.3.2.10 Protein reduction/alkylation and in-solution trypsin digestion

Trypsin digestion was performed as described in section **2.3.1.5**

2.3.2.11 Protein identification by nano-liquid chromatography and high- resolution tandem mass spectrometry (nanoLC-MS/MS)

The nanoLC-MS/MS analysis of the tryptic peptides was performed using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano HPLC system (ThermoFisher Scientific, USA) as described in section **2.3.1.6**

2.3.2.12 Database searching and criteria for protein identification

RAW data files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 (http://www.maxquant.org/) using the extracted ion currents of matched peptides (Cox *et al*. 2011) and proteins identified as described in section **2.3.1.7**

2.3.2.13 Determination of relative protein abundance, functional and bioinformatics analyses

MaxLFQ was used for peptide intensity determination and normalization, based on pair-wise comparison of unique and razor peptide intensities and a minimum ratio count of 2. The ProteinGroups output file generated by MaxQuant was imported into Perseus software

version 1.5.031 (http://perseus-framework.org/) and processed as described in section **2.3.1.8**. Proteins were filtered to include proteins detected in a minimum of 3 replicates of one treatment group unless otherwise specified. Missing values (reported as NAN and equivalent to zero LFQ intensity) were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances where applicable. Z-scored normalized protein expression matrices were generated by calculating mean LFQ peptide intensities and standard deviations for each sample. To determine proteins that were differentially modulated, a two-sided *t*-test with a permutation-based FDR of 0.05 was applied using 250 randomizations and a stabilization parameter s_0 of 0.5. Identification of proteins specific to a particular extract or growth temperature was done before imputing the missing values (reported as NAN and equivalent to zero LFQ intensity). All changes in relative expression of the proteins at different growth temperatures were determined against the control cultured at 30°C.

Functional analysis of the identified proteins was done based on protein Clusters of Orthologous Groups (COGs) (Tatusov *et al*. 2000) and KEGG (Kanehisa & Goto 2000) functional classification. Heat maps that depicted surface proteins which were differentially modulated in the LiCl-sucrose, trypsin shaving and ECF protein fractions at different tempratures were generated using excel. The presence of signal peptides was determined using SignalP 4.1 server with Gram-positive bacteria selected as organism group (Petersen *et al*. 2011). The default-cut-off values were used to optimize the performance measured as Matthews Correlation Coefficient (MCC) thereby resulting in higher true positive rate than SignalP 3.0. Positive prediction was considered true when a signal peptide was suggested according to the *D*-mean score of hidden Markov and networks-based models. Lipoproteins were those proteins predicted as lipid-anchored proteins via the Pred-Lipo server,

http://bioinformatics.biol.uoa.gr/PRED-LIPO/inputHelp.jsp, (Bagos *et al*. 2008). Subcellular transmembrane localization of the proteins was predicted using TMHMM server 2.0, http://www.cbs.dtu.dk/services/TMHMM/, (Krogh *et al*. 2001). Other subcellular localization predictions were performed by cell-Ploc 2.0, http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/, (Chou & Shen 2010) and PSORTb 3.0, http://www.psort.org/psortb/, (Nancy *et al*. 2010). MoonProt database, http://www.moonlightingproteins.org, was used to confirm identities of moonlighting proteins.

2.3.3 Materials and methods used in Chapter 5

2.3.3.1 Bacterial growth conditions

Lactobacillus casei GCRL163 was stored and prepared as previously described in section **2.3.1.1**.

2.3.3.2 Mid-exponential and stationary growth phases in bioreactors

Two water-jacketed Benchtop bioreactor units (New Brunswick, Eppendorf, USA), containing 800 mL of MRS broth, were inoculated to an OD_{600} of 0.02 from the starter culture. The cultures in the bioreactors were grown at 30°C and the anaerobic growth condition of the bioreactors maintained as previously described in section **2.3.1.2**. Samples were taken at intervals and the OD_{600} measured to monitor the growth. Samples for protein extraction were collected from a bioreactor unit at mid-exponential (6 h) and from the other bioreactor unit at stationary (24 h) growth phases. Three technical replicates were harvested from each bioreactor unit for preparation of protein fractions for proteomic analyses.

2.3.3.3 Cell surface protein extracts by lithium chloride-sucrose

The cell surface proteins from 100 mL of mid-exponential and stationary cultures were extracted by lithium chloride-sucrose as described in section **2.3.2.3**.

2.3.3.4 Protein extraction by trypsin shaving

The cell pellets from 100 mL of mid-exponential and stationary growth phases were washed once with 20 mL Tris-HCl buffer (0.04M, pH 6.8) and trypsin shaving of the whole cells performed as described in section **2.3.2.4**.

2.3.3.5 Extracellular culture fluid protein precipitation by trichloroacetic acid-acetone

The extracellular culture fluid from 100 mL of mid-exponential and stationary growth phase cultures were filtered through a 0.22μm nitrocellulose membrane and concentrated to 2.5% of the original volume using Amicon stirred cells with PLBC ultrafiltration discs, generated cellulose, 3000 NMWL, 63.5mm filter diameter (Merck PL, Australia). The precipitation of the proteins was performed as described in in section **2.3.2.5**. All protein concentrations were determined using the Bradford assay (Kruger 2002).

2.3.3.6 Protein reduction/alkylation and in-solution trypsin digestion

Trypsin digestion for nano-liquid chromatography and high-resolution tandem mass spectrometry (nanoLC-MS/MS) analysis was performed following described procedures in section **2.3.1.5**.

2.3.3.7 Protein identification by nanoLC-MS/MS

The nanoLC-MS/MS analysis of the tryptic peptides was performed using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano HPLC system (ThermoFisher Scientific, USA) as described in section **2.3.1.6**.

2.3.3.8 Database searching and criteria for protein identification

RAW data files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 (http://www.maxquant.org/) using the extracted ion currents of matched peptides (Cox *et al*. 2011). The extracted MS/MS spectra were searched against the UniProt proteome for *L. casei* strain W56 (ID UP000003734) database and proteins identified as previously described in section **2.3.1.7**.

2.3.3.9 Determination of relative protein abundance, functional and bioinformatics analyses

The *ProteinGroups.txt* output file generated by MaxQuant was imported into Perseus software version 1.5.031 (http://perseus-framework.org/) and processed as described in section **2.3.1.8**. Proteins were filtered to include proteins detected with at least two unique peptides. Identification of uncharacterized protein homologs was performed as described in section **2.3.1.8**.

Relative change in abundance of the proteins detected at mid-exponential and stationary growth phases was determined by comparing the mean $\log_2$-transformed label-free quantitation (LFQ) intensities. LFQ values were considered significant at $\log_2$-fold threshold of ≥ 1 (upregulation) or ≤ -1 (downregulation), ($p < 0.05$, FDR <5%). Moreover, proteins with mean $\log_2$-transformed LFQ intensities ≥ 23 were considered significant ($p < 0.05$, FDR <5%) for proteins detected in only one growth phase in different fractions (essentially being

undetected in one growth phase but detected at moderately high abundance at the other). Due to an absence of internal standards to adjust LFQ during analyses, LFQ values were adjusted by applying a common factor where necessary. We considered the average difference in LFQ values for all of the proteins that were common across the growth phases for a particular fraction. This determined the average relative upshift or downshift in LFQ against which proteins showing a higher change could be recognized. The fraction which showed the greatest upshift in LFQ was in the ECF stationary versus mid-exponential, where the mean LFQ was increased by $\log_2 4$. Adjustment was done by deducting the common factor $\log_2 4$ from the mean LFQ values at stationary growth phase. Subcellular localization of proteins was performed as described in section **2.3.2.13**.

2.3.4 Material and methods used in Chapter 6

2.3.4.1 Bacterial strain and prolonged heat stress conditions

Cheese isolate *L. casei* GCRL163 cultures used for this study was prepared as previously described in section **2.3.1.1**. Prolonged heat stress conditions were as previously described in section **2.3.1.2**.

2.3.4.2 Preparation of the cell-free extracts

This was done as previously described in section **2.3.1.3**, using 100 mL of mid-exponential growth phase at 30 to 45°C growth temperatures.

2.3.4.3 Extraction of cell-surface proteins by lithium chloride-sucrose

This was done as previously described in section **2.3.2.3**, using 100 mL of mid-exponential growth phase at 30 to 45°C growth temperatures.

2.3.4.4 Detection of cell-surface proteins by trypsin shaving

This was done as previously described in section **2.3.2.4**, using 100 mL of mid-exponential growth phase at 30 to 45°C growth temperatures.

2.3.4.5 Preparation of the extracellular fluid protein fractions by precipitation

Proteins in extracellular culture fluid from 100 mL of mid-exponential growth phase cultures at 30 to 45°C growth temperatures were precipitated as previously described in section **2.3.2.5**.

2.3.4.6 Protein reduction/alkylation and in-solution trypsin digestion

The trypsin digestion for nanoLC-MS/MS analysis was performed following described procedures in section **2.3.1.5**.

2.3.4.7 Protein identification by nano-liquid chromatography and high- resolution tandem mass spectrometry (nanoLC-MS/MS)

Tryptic peptides of each protein digest were analyzed by nanoLC-MS/MS analysis using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano system (ThermoFisher Scientific, USA) as previously described in section **2.3.1.6**.

2.3.4.8 Database searching and criteria for protein identification

RAW data files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 (http://www.maxquant.org/) using the extracted ion currents of matched peptides (Cox *et al*. 2011). The extracted MS/MS spectra were searched against the UniProt

proteome for *L. casei* strain W56 (ID UP000003734) database and proteins identified as previously described in section **2.3.1.7**.

2.3.4.9 Determination of relative protein abundance, functional and bioinformatics analyses

The *ProteinGroups.txt* output files generated by MaxQuant were imported into Perseus software version 1.5.031 (http://perseus-framework.org/) and processed as previously described in section **2.3.1.8**. Protein identification was performed using Uniprot, KEGG and NCBI as previously described in section **2.3.1.8**. Subcellular localization of proteins was done as described in section **2.3.2.13**.

The identification of the polymerase family proteins and alternative sigma factors on the genome of *L. casei* GCRL163 was performed by searching the Rapid Annotations using Subsystems Technology (RAST) annotated file of the genome sequence of *L. casei* GCRL163 (Nahar *et al*. 2017) and the Integrated Microbial Genomes (IMG) system. A forensic analysis of CFE, surface-associated proteins obtained by LS and TS and proteins collected from the ECF of *L. casei* GCRL163, was performed on LFQ proteomics datasets deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al*. 2015) partner repository with the dataset identifier PXD007097 for CFE and represented a filtered dataset of 859 proteins, which included proteins detected in a minimum of three replicates of any biological sample. Similarly, LS and TS proteomic data archived with dataset identifiers PXD009591 and PXD009468, representing 826 and 183 filtered proteins respectively, and the ECF dataset identifier PXD009638, which represented a filtered dataset of 47 proteins, were used. Key regulators associated with the HSR involved in PHSR were identified from the proteomic datasets and other proteins potentially involved in PHSR were identified based

on their DNA- and RNA-binding properties, or involvement in a variety of functions including signal transduction, modulation of cytokinesis, transcription, translation and post-translational modification.

2.3.5 Material and Methods used in Chapter 7

2.3.5.1 Bacterial strain and growth conditions

Storage and growth conditions of *L. casei* GCRL163 are as described in section **2.3.1.1**.

2.3.5.2 Prolonged heat stress conditions

L. casei GCRL163 was anaerobically cultured to mid-exponential growth phase in strictly controlled bioreactor system as previously described in section **2.3.1.2**. The growth temperatures were maintained at 30°C, 40°C and 45°C and the pH was set at 6.5 by online addition of 2M NaOH.

2.3.5.3 Preparation of the cell free, surface protein and extracellular fluid extracts

The CFEs were prepared as previously described in section **2.3.1.3**.

Cell surface protein fraction was obtained by lithium chloride-sucrose and trypsin shaving of the intact cells as previously described in sections **2.3.2.3** and **2.3.2.4** respectively.

The ECF protein fraction was obtained by filtering the cell-free supernatant fluid through 0.22μm nitrocellulose membranes and prepared as previously described in section **2.3.2.5**.

2.3.5.4 Protein reduction/alkylation and in-solution trypsin digestion

The trypsin digestion for nanoLC-MS/MS analysis was performed as previously described in section **2.3.1.5**.

2.3.5.5 Protein identification by nano-liquid chromatography and high- resolution tandem mass spectrometry (nanoLC-MS/MS)

Tryptic peptides equivalent to 1 µg of each protein digest were analyzed by nanoLC-MS/MS analysis using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano system (ThermoFisher Scientific, USA) as previously described in section **2.3.1.6**.

2.3.5.6 Database searching and statistical analysis of identified proteins

RAW data files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 (http://www.maxquant.org/) using the extracted ion currents of matched peptides (Cox *et al*. 2011). The extracted MS/MS spectra were searched against the UniProt proteome for *L. casei* strain W56 (ID UP000003734) database and protein identified as previously described in section **2.3.1.7**. Proteins were filtered to include proteins identified with at least two unique peptides and detected in a minimum of three replicates of any biological sample. Where necessary, missing values were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances. To determine proteins that were significantly altered in abundance by heat treatments, a two-sided *t*-test using Benjamini Hochberg correction for multiple hypothesis testing was applied. A 0.5% FDR threshold was applied to determine statistical significance. Protein identification and subcellular localization of proteins were performed as previously described in sections **2.3.1.8** and **2.3.2.13**. The proteomic datasets archived at the ProteomeXchange Consortium

via the PRIDE (Vizcaíno *et al*. 2015) partner repository with the dataset identifiers PXD007097, PXD009591, PXD009468 and PXD009638 for CFE, LS, TS and ECF respectively and filtered as described in section **2.3.4.9** were used to determine change in protein abundance in the protein fractions. All the metabolic maps were adapted from KEGG pathways (Kanehisa & Goto 2000) and the construction of the maps was based on two-sample-*t*-test difference of $\log_2$-transformed label-free quantitative proteomic data from cells grown under controlled cultured conditions at different temperature relative to 30°C.

CHAPTER 3

PROTEOMIC ANALYSIS OF *LACTOBACILLUS CASEI* GCRL163 CELL-FREE EXTRACTS REVEALS A SECB HOMOLOG AND OTHER BIOMARKERS OF PROLONGED HEAT STRESS

A manuscript published in *PLOS ONE* was adapted from this Chapter.

3.1 Abstract

Prolonged heat stress is one of the harsh conditions *Lactobacillus casei* strains encounter as non-starter lactic acid bacteria in dairy product manufacture. To understand the physiological and molecular mechanisms through which *L. casei* GCRL163 adapts to persistent elevated temperature, label-free quantitative proteomics of cell-free extracts was used to characterize the global responses of the strain cultured anaerobically in bioreactors at 30 to 45°C, pH 6.5, together with GC-MS for fatty acid methyl ester analysis at different growth phases. At higher growth temperatures, repression of energy-consuming metabolic pathways, such as fatty acid, nucleotide and amino acid biosynthesis, was observed, while PTS- and ABC-type transporter systems associated with uptake of nitrogen and carbon sources were up-regulated. Alkaline shock protein Asp23_2 was only detected at 45°C, expressed at high abundance, and presumptive α-L-fucosidase only at 40 and 45°C, with highly increased abundance (log_2-fold change of 7) at 45°C. We identified a novel SecB homolog as a protein export chaperone putatively involved in post-translational translocation systems, which was down-regulated as growth temperature increased and where the modelled 3D-structure shared architectural similarities with the *Escherichia coli* SecB protein. Membrane lipid analyses revealed temporal changes in fatty acid composition, cyclization of oleic acid to cyclopropane and novel cyclopentenyl moieties,

and reduced synthesis of vaccenic acid, at higher temperatures. An 18kDa α-crystallin domain, Hsp20 family heat shock protein was more highly up-regulated in response to heat stress compared to other molecular chaperones, suggesting this protein could be a useful biomarker of prolonged heat stress in *L. casei* GCRL163.

3.2 Introduction

Lactobacillus casei and related species occur in fermented milk and cheese products as non-starter, adventitious microbiota where they improve ripening and flavour development (Kourkoutas *et al*. 2006). Benefits to human health, demonstrated in animal models (Kapila & Sinha 2006; Wang *et al*. 2016), have been attributed to ingestion of appropriate probiotic formulations containing *L. casei*. However, during probiotic formulation, manufacture of fermented food and passage through the gastrointestinal tract, bacteria are exposed to several potential stressors, such as high or low temperature, starvation, low pH, bile salts, and changing redox and osmotic conditions (Ferrando *et al*. 2015; Papadimitriou *et al*. 2016; Rossi *et al*. 2016). Heat stress is one of the most characterized and commonly encountered conditions experienced by various lactic acid bacteria (LAB) (Di Cagno *et al*. 2006). For instance, LAB in raw milk are exposed to high temperatures during pasteurization and starter cultures added for the production of different types of cheese are also subjected to heating 9cooking and scalding) after pasteurization (Papadimitriou *et al*. 2016). To confer probiotic and other functional traits, LAB must survive exposure to heat and other stressors during manufacturing processes, as well as harsh conditions encountered in the gastrointestinal tract following consumption. Under these conditions, several physiological and molecular responses are induced to enhance growth, adaptation and survival so alleviating impacts of stress (Papadimitriou *et al*. 2016).

Heat stress responses have often been studied as a rapid transient upshift in temperature whereby bacterial populations are exposed briefly to heat shock, eliciting the heat shock response which is commonly used to describe adaptive responses of bacteria to heat stress (Broadbent *et al*. 1997; De Angelis *et al*. 2004; Tripathy *et al*. 2016). The heat shock response is typified by the upregulation of chaperone proteins involved in protein folding and turnover (Gottesman *et al*. 1997; Sugimoto & Sonomoto 2008). Chaperone proteins are also induced by other stressors and prior heat shock pre-conditions cells to protection against subsequent challenge by other stressors (De Angelis *et al*. 2016; De Angelis & Gobbeti 2004). In practise, however, LAB populations are typically exposed to prolonged periods of heating during fermented food processing. For instance, pasteurization of raw milk involves exposing the microbiota, including LAB, to high temperatures (>60°C) for up to 30 min (Papadimitriou *et al*. 2016), resulting in lowered bacterial load. The cellular and physiological responses of *E. coli* and *B. thuringiensis* YBT-1550 to such prolonged heat stress have been reported (Lüders *et al*. 2009a; Wu *et al*. 2011). Studies of heat stress at a transcriptional level involving *B. breve* UCC 2003 for 150 min has also been reported (Ventura *et al*. 2004). However, there is a paucity of detailed information available at the transcriptomic or proteomic level on how LAB cells, in particular *L. casei*, respond to prolonged period of stress, including heat stress, and whether the underlying mechanisms differ from 'shock' responses. Indeed, there is growing research interest in documenting the stress physiology of LAB species under long-term exposure to stressors in context of improving the fitness of strains for fermentation and probiotic applications (Papadimitriou *et al*. 2016; Zhang *et al*. 2018). Improvements in proteomic technologies, moving from two-dimensional-gel/mass spectrometry (MS) to high-throughput, gel-free, MS/MS systems, has greatly improved proteomic analyses, particularly for determining modulation of proteins

expressed in low abundance, expanding the current knowledge of stress adaptation in *Lactobacillus* (De Angelis *et al*. 2016).

In the current investigation, we identified changes in the cellular proteome of *L. casei* GCRL163 cultured under anaerobic, pH-controlled conditions in bioreactor systems for culture temperatures of 30°C, 35°C, 40°C and 45°C. Label-free quantitative proteomic analysis of cell free extracts (CFEs) from mid-exponential cultures and statistical analysis revealed that an increasing number of proteins were affected as the growth temperature was increased. More than 50% of the identified proteins were significantly modulated during prolonged heat stress experienced during culture at a temperature close to the maximum permissible growth temperature of 45°C, suggesting that this strain depended on the mobilization of heat stress machinery vital for protecting cellular and metabolic functions to initiate and sustain growth. Moreover, metabolic pathways appeared to be rerouted during extreme thermal stress to conserve energy, with repression of energy-consuming activities, such as fatty acid and nucleotide biosynthesis, and upregulation of enzymes in the glycolytic pathway at 45°C. In contrast, cells cultured at 40°C induced responses for maintaining cellular survival, including upregulation of most proteins in fatty acid synthesis and non-differential modulation of most proteins involved in glycolysis. Changes in fatty acid composition were observed to be growth phase- and temperature-dependent as previously reported in other bacterial species (Broadbent *et al*. 2010; Nagamachi *et al*. 1991) with reduced vaccenic acid synthesis in cells from 45°C culture and detection of cyclopropane derivatives of C18:0 fatty acids and previously unreported cyclopentenyl moieties.

3.3 Results

3.3.1 Quantitative and bioinformatic-functional analysis of differentially expressed proteins during prolonged heat stress

According to growth curves obtained for temperatures between 25°C and 55°C, the highest growth rate for strain GCRL163 was at 37°C and no significant growth occurred at temperatures above 50°C (Figure 3. 1). When cultured in bioreactors at 45°C, the μ_{max} was greatly reduced relative to lower temperatures and cells entered stationary phase earlier, with a lower final biomass (judged from OD_{600} readings), indicating considerable metabolic impairment of cells growing close to the maximum permissible temperature for this strain, 45°C (Figure 3. 1). To investigate the response of *L. casei* GCRL163 to prolonged exposure to growth-permissive but inhibitory temperatures at the proteome level, we used the MaxQuant platform for label-free quantitative (LFQ) proteomic analysis (see Materials and Methods). The peptide- and protein-level MaxQuant output files are reported in Supplementary Table 3. 1. A high-confidence dataset, comprising of proteins detected in a minimum of 9 of the 12 biological samples and excluding proteins with <2 matching peptides suspected contaminants and reverse database matches, was used for statistical comparison of the treatment groups. Of the 1,081 total proteins identified in the cell-free extracts, 773 proteins met these filtering criteria. Principal component analysis indicated PC1 explained 65% of the variance in the data, with increasing separation of the higher temperature samples from the 30°C samples (Figure 3. 2A). Consistent with this, two-sample *t*-tests indicated that an increasing number of proteins were significantly impacted at increasing temperatures. For example, at a FDR threshold of 1%, 3 proteins, 285 proteins and 515 proteins were significantly affected at 35°C, 40°C and 45°C, respectively, relative to growth at 30°C (Figure 3. 2B-D). This result suggests that growth temperatures of 40°C and 45°C impose significant thermal stress on *L. casei* GCRL163.

The results of statistical analysis of the protein LFQ expression data are shown in full in Supplementary Table 3. 2, and the most highly differentially expressed proteins, according to the fold-differences in mean protein LFQ values at 35°C, 40°C and 45°C, respectively, relative to 30°C, are labelled numerically on the *t*-test volcano plots and summarized in Table 3. 1.

To gain a global overview of the protein functional classes impacted by thermal stress, proteins in the filtered set of 773 were grouped according to GO categories for molecular function based on the functional annotations of strain *L. casei* W56 and changes in abundance relative to 30°C were analysed using T-profiler (Supplementary Table 3. 3). Clustering the data according to *t*-values revealed protein functional classes with consistent trends across all three elevated temperatures (either consistently up- or down-regulated) and those with more complex patterns of regulation (Figure 3. 3). For example, proteins associated with the terms membrane bioenergetics (*n*=12) and protein folding and turnover (*n*=22) were consistently more abundant at growth temperatures of 35°C, 40°C and 45°C relative to 30°C. In contrast, proteins involved in phosphotransferase systems (*n*=19) and ABC-type transporter systems (*n*=38) were up-regulated only at 40°C and 45°C, while carbohydrate-related metabolism (*n*=37) and central glycolytic and intermediary pathways (*n*=29) were enhanced specifically at 45°C. Of the protein functional classes that were consistently repressed at 35°C, 40°C and 45°C, the largest groups of proteins were associated with the terms amino acid-related metabolism (*n*=46) and nucleic acid/nucleotide metabolism (*n*=50). Functional classes with mixed responses to thermal stress at different temperatures included proteins associated with DNA repair/recombination (*n*=16) and co-factor related metabolism (*n*=28), which were down-regulated at 40°C and 45°C and proteins involved in lipid-related metabolism (*n*=27), which overall were attenuated at 45°C specifically.

3.3.2 Qualitative analysis to reveal proteins specific to the stress temperatures

The statistical analysis described above included several proteins that were absent from all three replicates at one specific temperature but detected in all replicates of the three other conditions, and therefore apparently repressed in a temperature-specific manner. From this, we might speculate that proteins that are suppressed, or uniquely expressed, at one or more specific growth temperature (s) could provide insight into survival strategies during heat prolonged heat stress. Of these proteins, 33 were detected at moderate to high abundance (average LFQ values) in all the 30°C, 35°C and 40°C CFE samples but not detected at 45°C, and one protein (ThiM) expressed at growth temperatures of 35°C, 40°C and 45°C was not detected at 30°C (Table 3. 2). In addition, based on the complete set of proteins identified in the study, temperature-specific expression patterns were identified in proteins detected in 6/12 samples and 3/12 samples (Supplementary Table 3. 4, Table 2). These included proteins detected at 30°C and 35°C only (i.e. apparently suppressed at 40°C and 45°C), notably several proteins involved in purine biosynthesis, and conversely, three proteins that were detected only at 40°C and 45°C. Finally, a group of five proteins was found at one specific temperature only, which included one protein specific to 45°C (Asp23_2, alkaline shock protein BN194_01950) and four proteins specific to 40°C. The proteins specifically expressed at 40°C and 45°C include proteins involved in sugar uptake, methionine or galactitol uptake and uncharacterized proteins that might participate in α-L-fucosidase activity, glucan-like efflux, toxic substance efflux and general stress responses. The α-L-fucosidase was detected at $\log_2$-fold 7 higher at 45°C than at 40°C, indicating significantly higher protein expression at this elevated temperature (Supplementary Table 3. 2).

3.3.3 Expression level of molecular chaperones and other proteins associated with protein misfolding are modulated in *L. casei* GCRL163 at elevated growth temperature

The protein functional class 'protein folding and turnover' showed higher protein expression levels at all temperatures above 30°C and notably higher overall changes in abundance at 45°C (Figure 3. 3, Figure 3. 4A). However, there were considerable differences in the abundance of the proteins detected and the degree of change in abundance (LFQ values) for individual proteins (Figure 3.4A, Figure 3. 5). With the exception of DnaJ and HcrA, the molecular chaperones specified in the GroEL-GroES and HcrA-GrpE-DnaK-DnaJ gene regions, proteins involved in nascent protein folding during normal growth and in heat shock responses in many bacteria (Hartl 1996), were highly abundant proteins at all temperatures. GroEL and GroES were most highly induced at 45°C, by $\log_2$ 3.6-fold and 3.1-fold, respectively (FDR<1%), with relatively small fold-changes at 35°C and 40°C, DnaK and GrpE followed similar trends. The heat-inducible regulator, HcrA, was detected in low abundance which was not significantly altered in CFEs at different temperatures (Supplementary Table 3. 2). DnaJ was also present in low abundance at 30°C and was up-regulated at all temperatures above 30°C (increased $\log_2$ 1.7-fold, 2.1-fold and 3.3-fold at 35°C, 40°C and 45°C, respectively), showing a different regulatory pattern than the other chaperones. From the published genomes, intergenic regions were detected upstream of the *hrcA*, *grpE*/*dnaK* and *dnaJ* genes in strain GCRL163 and other *L. casei* strains examined (MJA12, ATCC 334, ATCC 393, W56) and *L. paracasei* ATCC 25302, although the length of the regions varied in some strains: the BPROM tool detected one promoter in the 112 bp intergenic region upstream of *dnaJ* in all strains except W56. This suggested that the regulation of *dnaJ* may differ between strains and that separate regulation of the genes in this cluster may occur, consistent with the observed proteomic data.

An 18kDa α-crystallin domain heat shock protein (gene locus BN194_29440), which belongs to the small heat shock protein (Hsp20) family, was in very low abundance at 30°C but this was more highly increased with temperature compared with the other heat shock proteins (increased $\log_2$ 3.1-fold, 5.4-fold and 7.2-fold at 35°C, 40°C and 45°C respectively) (Figure 3. 5). The degree of upregulation of this protein at temperatures close to the optimum for growth for this strain may suggest a role in inhibiting protein aggregation in response to increases in growth temperature. Other small HSPs (for example Hsp33) were not similarly altered in abundance with increased culture temperature (Figure 3. 5).

The majority of other heat-inducible proteins, including the Clp family of ATP-dependent proteases, are involved in recycling defective proteins (Kilstrup *et al*. 1997): ClpB, ClpC and ClpE were also strongly induced at 45°C (by $\log_2$ 2.1-fold, 3.1-fold and 2.2-fold, respectively). Other proteins associated with stabilization or degradation of misfolded/unfolded proteins, such as the foldase PrsA and the serine protease HtrA, were significantly enhanced at both 40°C and 45°C (FDR<1%). With the exception of PepC_2, which was more highly abundant at 40°C (Supplementary Table 3. 2), other peptidases were not modulated following growth at different temperatures.

3.3.4 Expression of proteins in transport systems affected by heat stress

The protein expression data for proteins within the ATP-binding cassette (ABC) transporter systems also revealed a complex pattern of protein modulation across the different temperature treatments, as shown in Figure 3. 4B. A high proportion of these proteins were significantly up-regulated at both 40°C and 45°C relative to 30°C, with maximal expression changes typically detected at 40°C. This applied to several proteins involved in polar amino acid uptake, notably several proteins involved in glutamine uptake including GlnH, GlnH4,

GlnQ3 and GlnQ4 (increased $\log_2$ 2.8-fold, 1.6-fold, 2.3-fold and 1.2-fold, respectively); proteins involved in polyamine uptake such as PotD and PotA_2 ($\log_2$ 3.5-fold and 2.6-fold increased, respectively); and toxic substance efflux (YfiB, $\log_2$ 3.3-fold increased). OpuCA, a protein involved in glycine-betaine uptake, and 2 proteins with putative ABC transporter functions (permease, BceA-type transmembrane protein BN194_21220 and ATP-binding protein BceA_2) were most highly induced at 45°C relative to 30°C (increased $\log_2$ 2.4-fold, 3.9-fold and 3.0-fold, respectively). BN194_21220 also possesses a FtsX-like domain and BN194_21230 an AAA+ ATPase domain (Consortium 2016), which may suggest that these proteins are involved in inserting proteins into cytoplasmic membranes.

At higher growth temperatures, some proteins that facilitate oligopeptide uptake were repressed, whereas others were increased. OppA2, OppD2 and OppF2 were significantly repressed at both 40°C and 45°C relative to 30°C (all FDR<1%, except OppA2 at 45°C, FDR<5%). In contrast, the oligopeptide transport system permease OppC and oligopeptide transport ATP-binding protein OppD were significantly increased at both 40°C and 45°C relative to 30°C (FDR<1% for both proteins at 40°C and FDR<5% for OppC at 45°C) while OppA was elevated at 40°C only ($\log_2$ 1.84-fold, FDR<1%). These proteins could assist *L. casei* GCRL163 accumulate oligopeptides from which amino acids are generated through the activity of peptidases including HtrA from degradation of peptidoglycan. Although proteins in the cell wall biogenesis functional class were largely unchanged at higher temperatures in the CFE (see Figure 3. 3), both ManD and NagA, which are involved in degradation of peptidoglycan through the conversion of N-acetyl-glucosamine-6-phosphate into D-glucosamine-6-phosphate thence fructose-6-phosphate plus glutamine, were up-regulated at 45°C (Figure 3. 4C), supporting recycling of muropeptides through peptidoglycan turnover (Johnson *et al*. 2013).

In addition to alterations in amino acid and oligopeptide transporters, most of the proteins included within the functional class 'phosphotransferase (PTS) system' were significantly induced at elevated temperatures (Supplementary Table 3.2). These included FruA3, BglP and SorB_2 which were associated with fructose, β-glucoside and sugar uptakes respectively and enhanced at 40°C and 45°C. Other up-regulated PTS proteins are ManY/LevF (log_2 2.1-fold at 40°C and 1.2-fold at 45°C), ManY (log_2 3.1-fold at 40°C), LevG/ManZ (log_2 2.2-fold at 40°C and 1.0-fold at 45°C), ManZ (log_2 3.0-fold at 40°C, FDR<1% and 1.6-fold at 45°C, FDR<5%) and LevE/ManX (log_2 1.4-fold at 40°C, FDR<5%). However, repression of some PTS proteins was also observed and they include uncharacterized protein BN194_04820 (decreased log_2 2.2-fold at 40°C and 45°C) associated with uptake of mannose, fructose or sorbose, MtlA (log_2 3.0-fold at 45°C), Lev_3 (log_2 2.3-fold at 45°C) and ManX (log_2 4.8.0-fold at 45°C). Lev_4 involved in sugar uptake was specifically expressed at 40°C and 45°C while TagT associated with galactitol uptake was expressed only at 40°C (see Table 3. 2). Several of the PTS proteins are annotated as components of mannose/fructose/sorbose uptake systems but recognized as having broad sugar transport capacity (Postma *et al*. 1993) including amino- and nucleotide-sugars. The core PTS proteins, including HprK (increased log_2 1.4.0-fold at 45°C), Ptsl (decreased log_2 1.3.0-fold at 45°C) and PtsH (decreased log_2 1.2.0-fold at 40°C, FDR<5%), are impacted by thermal stress.

3.3.5 Prolonged heat stress enhanced modulation of proteins involved in carbohydrate metabolism

As a fermentative bacterium when cultured under anaerobic conditions, *L. casei* GCRL163 must obtain energy by substrate-level phosphorylation (Konings, WN

2002). Glucokinase (GlcK), which was enhanced at 40°C (log_2 1.1-fold) and 45°C (log_2 2.4-fold), might be responsible for the conversion of glucose to glucose 6-phosphate in the bacterial strain with ATP being consumed, during glycolysis (Figure 3. 4C). Similarly, 6-phosphofructokinase catalysing fructose 6-phosphate to fructose 1, 6-biphosphate, which is an energy-consuming step in glycolytic pathway, was enhanced particularly at 45°C (log_2 1.3-fold). Several other enzymes associated with glycolysis were modulated during thermal stress, in particular at 45°C. These included phosphofructokinase (Pfk), enolase (Eno), fructose-bisphosphase aldolase (Fba2) and triosephosphase isomerase (Tpi), which were increased log_2 1.3-fold, 2.3-fold, 2.5-fold and 1.1-fold, respectively. Glyceraldehyde-3-phosphate dehydrogenase, which initiates the first substrate-level phosphorylation in glycolysis (through the conversion of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate) and phosphoglycerate kinase (Pgk) that dephosphorylates 1, 3-bisphosphoglycerate to generate 3-phosphoglycerate and ATP, were significantly up-regulated after growth at 45°C (log_2 1.7-fold and log_2 2.6-fold respectively). However, Pyk and Fba2 were both repressed at 40°C compared to 30°C.

ATP can be obtained through the metabolism of pyruvate to acetate in the pyruvate metabolism pathway (Konings *et al*. 1997). Pyruvate is converted to acetyl-P by Pta and Pox5, then acetyl-P is converted to acetate by AcyP (which is a phosphatase and does not produce energy) and AckA (which does produce energy by substrate level phosphorylation). The majority of enzymes that generate acetate from pyruvate were repressed at 45°C but were more abundant at 40°C relative to 30°C (Figure 3. 4C).These included pyruvate oxidase Pox5 (reduced log_2 2.0-fold), two acetate kinases AckA and AckA_2 (reduced log_2 1.9-fold and log_2 1.2-fold, respectively) and the acylphosphatase AcyP (reduced log_2 1.7-fold), while Pta and L-Ldh were moderately enhanced (log_2 0.9-fold) at the elevated temperature of 45°C.

Pyruvate carboxylase and α-oxaloacetate decarboxylase (responsible for the synthesis or degradation of oxaloacetate from pyruvate, respectively), malate dehydrogenase and formate acetyltransferase (pyruvate formate lyase, Pfl) were all inhibited (Figure 3.4C, Supplementary Table 3.2). Three subunits of the pyruvate dehydrogenase complex (PdhA, PdhB and PdhC) were all increased between $\log_2$ 1.0-fold to 1.4-fold at elevated temperatures while PdhD was enhanced only at 40°C relative to 30°C. Aldehyde-alcohol dehydrogenase (AdhE) was enhanced at 45°C ($\log_2$ 3.5-fold, FDR<1%), but repressed at 40°C ($\log_2$ 2.9-fold, FDR <5%). AdhE can also participate in metabolic pathways involving metabolite biosynthesis, tyrosine and butanoate metabolism, ethanol generation and lipid degradation (Kanehisa & Goto 2000). Furthermore, the ATP synthase subunits α (AtpA), β (AtpD), b (AtpF), γ (AtpG) and δ (AtpH), associated with membrane bioenergetics, were all up-regulated at 40°C and 45°C. Overall, these results indicate that *L. casei* GCRL163 induces a complex network of metabolic processes to ensure adequate energy production to meet the physiological demands associated with growth and survival under thermal prolonged heat stress.

3.3.6 Heat stress repressed expression of proteins associated with fatty acid metabolism

Proteins involved in lipid-related metabolism that were identified in the current study included enzymes involved in fatty acid biosynthesis that were increased at 40°C, while most were repressed during growth at 45°C (Figure 3. 4D). This could reflect the need to conserve cellular metabolic energy by limiting energy-demanding processes involved in fatty acid biosynthesis. For instance, members of the Fab protein family that were repressed at 45°C included FabD, FabF, FabG, FabH, FabK and two FabZ isoforms (repressed $\log_2$ 5.4-fold, 1.7-fold, 2.4-fold, 2.1-fold, 1.1-fold, 4.91-fold and 5.3-fold, respectively) while FabG and one FabZ isoform were increased at 40°C ($\log_2$ 1.1-fold, FDR<5% and $\log_2$ 1.2-fold, FDR<1%,

respectively) (Figure 3. 6). Similarly, the acetyl–CoA carboxylase carboxyl carrier protein AccC was repressed at 45°C (log_2 3.3-fold) but enhanced at 40°C (log_2 1.6-fold). An AccC isoform (BN194_22500) was also identified to be up-regulated at 40°C (log_2 1.6-fold, FDR<5%). Glycerol 3-phosphate acyltransferase PlsX, associated with phospholipid biosynthesis, was the only protein up-regulated at both 40°C and 45°C (log_2 1.36-fold, and 2.0-fold, respectively). Oleate hydratase Sph was repressed across the growth temperatures by log_2 1.3-fold at 35°C (FDR<5%) and log_2 1.6-fold at 40°C and was suppressed beyond detection at 45°C. MvaS, involved in isoprenoid biosynthesis in the mevalonate pathway and bifunctional protein BirA were repressed at 40°C and 45°C, while repression of the acyl carrier protein AcpP2 was observed only at 45°C (log_2 3.1-fold).

We also identified cyclopropane fatty acid acyl synthase (BN194_22460), a methyl-transferase putatively involved in the synthesis of cyclic fatty acid cyclopropane (which is in gene neighbourhood of the fatty acid synthesis genes), that was most highly induced at 35°C (log_2 1.1-fold, FDR<5%) with lesser increases at 40°C and 45°C (log_2 ~0.7-fold, FDR<5%). Since changes in membrane fatty acid composition have been associated with heat stress in lactobacilli (Russell *et al*. 1995), we therefore investigated the relative percentages of the cell fatty acids with respect to growth phase and temperature of growth (Table 3. 3).

3.3.7 Impact of prolonged heat stress on fatty acid composition

In this study, the principal unsaturated fatty acid was *cis*-9-octadecenoic acid (oleic acid) while the dominant saturated fatty acid was hexadecanoic acid (palmitic acid). Formation of palmitic acid increased from exponential to stationary phases in cells grown at 30 to 40°C (Table 3.3). Furthermore, in mid-exponential phase, the proportion of palmitic acid increased from 15.1% at 30°C to 26.4% at 45°C. The proportion of oleic acid detected

decreased as the cells aged from exponential to stationary phases and also with increase in growth temperature. At 30°C, the proportion of oleic acid decreased from 66.4% at mid-exponential phase to 33.0% at stationary phase was observed, with concomitant increases in vaccenic acid. Similar trends were noted in cells grown at 35°C and 40°C across the growth phases. Reduction in the proportion of oleic acid from 66.4% at 30°C to 50.2% at 45°C in mid-exponential phase was also observed. Synthesis of *cis*-11-octadecenoic acid (vaccenic acid) was observed to be growth phase- and temperature-dependent, as production increased from exponential to stationary phase and across the growth temperatures. Two isomers of cyclopropane FA (cyclopropane-18:0a and cyclopropane-18:0b) and novel cyclopentenyl moieties, four isomers of C18:0 (retention index values of 2144, isomer a; 2153, isomer b; 2170, isomer c and 2187 isomer d), formed which are likely products of oleic acid cyclization in the cells. Production of the cyclopentenyl moieties was, however, reduced at 45°C. Our data demonstrate that the phase of growth and culturing temperatures substantially modulate FA composition in *L. casei* GCRL163 and the cells are conserving energy by adjusting the existing fatty acids rather than synthesizing new ones.

3.3.8 Prolonged heat stress enhanced post-translational protein secretion systems and thiamine metabolism

Proteins associated with post-translational protein secretion belonged to a relatively small but important subgroup of proteins that were differentially expressed during thermal adaptation (Figure 3. 7). The cytoplasmic ATPase SecA, reported to channel precursor proteins into the SecYEG translocon channel used for preprotein translocation across the plasma membrane, and YajC encoding protein that participates in liberating the preproteins from the channel (Duong & Wickner 1997), were both enhanced at 40°C and 45°C, and the central *S*ecY subunit itself was detected at increased levels at 40°C (log_2 3.0-fold; FDR>5%)

and 45℃ (log_2 3.2-fold; FDR<5%). Upregulation of the signal peptidase LepB at both 40°C and 45℃ (log_2 1.8-fold and 1.9-fold, respectively; FDR<1%) was consistent with a role for LepB in the release of mature polypeptides (Schneewind & Missiakas 2014) from the channel under thermal stress. Several chaperones contribute to post-translational secretion in Gram-positive and Gram-negative bacteria, such as SecB (Randall 1992), heat shock proteins Dnak, DnaJ and GrpE (Wild *et al*. 1996) and also trigger factor Tig (peptidyl-prolyl isomerase) (Stoller *et al*. 1995). In our current study, we identified an uncharacterized 15 kDa protein with gene locus BN194_10000 with the domain of export chaperone SecB in *L. casei* GCRL163, which is conserved in several genomes of *L. casei* strains as documented on UniProt (Consortium 2016). This protein was maximally expressed at the control temperature of 30°C and significantly repressed at 40°C and 45°C (by log_2 2.3-fold and 4.1-fold, respectively) in *L. casei* GCRL163. Trigger factor Tig, which is a protein export chaperone that ensures newly synthesized proteins are kept in an open conformation, was not significantly induced during prolonged heat stress but was detected in CFEs at very high abundance (Supplementary Table 3.2). Up-regulated Foldase protein PrsA at elevated temperature is a chaperone protein associated with post-translocational folding of many secreted proteins extracellularly. These results suggest that increased secretion, especially through the post-translational system, may be one of the major changes involved in the survival of *L. casei* GCRL163 during prolonged heat stress.

Our proteomic data (Supplementary Table 3.2) further revealed that most proteins associated with cofactor-related metabolism were down-regulated at 40°C and 45°C, including proteins encoded by LplJ, PncB, SufB, SufC, FolA, ApbE and IscS. However, in stark contrast, the hydroxyethylthiazole kinase ThiM, which is associated with thiamine biosynthesis, was the most highly up-regulated protein at all three elevated temperatures (log_2

4.1-fold at 35°C, FDR<5%; 6.6-fold at 40°C and 7.3 at 45°C) (Figure 3. 2, Table 1). The biologically active derivative of thiamine, thiamine diphosphate, is an important cofactor for several enzymes associated with amino acid and carbohydrate metabolism (Kowalska & Kozik 2008).

3.3.8 The *L. casei* SecB homolog is phylogenetically distant from *E.coli* SecB but structurally similar

BLASTP (KEGG) of the *L. casei* GCRL163 SecB homolog (protein sequence from the RAST file of the sequenced genome (Nahar *et al*. 2017) showed 100% sequence similarity with *L. casei* and *L. paracasei* proteins while *L. rhamnosus* strains were 91.9-92.6% similar to the *L. casei* GCRL163 SecB homolog sequence (Supplementary Figure 3. 1). Although the *L. casei* and *L. rhamnosus* proteins were closely related and conserved across the strains reviewed, they formed separate clades and were distinct from the other *Lactobacillus* species. Other proteins with SecB domains were identified in UniProt searches for 'SecB' then by genus (*Escherichia*, *Bacillus*, *Lactobacillus*), and a selection of these were chosen for sequence similarity analysis: a reviewed entry representative of Gram-negative SecB proteins (*E. coli* K12 protein ID P0AG86, where all top 50 hits for *E. coli* were 100% identical to this protein); two proteins from Gram-positive species with a submitted but unreviewed name of 'Preprotein translocase subunit SecB' (*Bacillus* sp. CC120222-01 protein A0A1X7ETD2 and *L. plantarum* subsp. *plantarum* ATCC14917 protein D7V892); and an uncharacterised *Bacillus* protein with a domain structure similar to the *L. casei* domains (*B. licheniformis* CG-B52 uncharacterised protein T5HC28, with overlapping homologous domains DUF1149, SecB [IPR003708] and SecB-like superfamily [IPR035958]) (Supplementary Figure 3. 1). The presumptive SecB detected in *L. casei*

GCRL163 showed low sequence similarity with *E. coli* SecB (16.0%) whereas the *Bacillus* and, interestingly, *L. plantarum* subsp. *plantarum*, showed greater similarity (48-54%).

X-ray crystal structures of *E. coli* and *Haemophilus influenzae* SecB proteins (Zhou & Xu 2005) show a common structure consisting of 4 anti-parallel β-sheets (β1 and β2 are outer strands), a main α1-helix and C-terminal anti-parallel α2-helix and a crossover loop between β1 and β2, all of which have proposed functional roles in binding client proteins or binding to SecA (Huang *et al*. 2016). The 3D protein models for *E. coli*, *L. plantarum* subsp. *plantarum* and *B. licheniformis* showed these common structural features (Figure 3. 8A, E and F), supporting the contention that the uncharacterised proteins in the latter two species are functionally similar. However, the *L. casei* (W56, GCRL163) and *L. rhamnosus* GG models (Figure 3. 8B, C and D) lack the second C-terminal helix (although this region on all of the models had a low confidence score), and also possess a truncated β2-strand and a diminished crossover loop. Notwithstanding these differences, and the difference in protein sizes, when aligned against the *E. coli* protein (Figure 3. 8G-K), the overall architecture of all of the proteins was similar.

3.4 Discussion

Stress physiology of LAB has been reviewed extensively and, despite genomic differences and strain-to-strain variation in responses across and within species, and differences in experimental approaches employed, there are striking similarities in how this group of genera responds to stressors (Papadimitriou *et al*. 2016; De Angelis *et al*. 2016; De Angelis & Gobbeti 2004). Common global responses include protection of macromolecules involved in metabolic activities (notably through chaperones engaged in protein folding and turnover) or cellular structural integrity (cell envelope components, including membrane

lipids); modulation of metabolic activity and energy production to ameliorate the impact of stress, typically around the fate of pyruvate; altered uptake of alternative carbon sources to facilitate increased energy production; and increased proteolysis and catabolism of amino acids. While growth in bioreactors at 40°C was marginally impaired, 45°C caused significant decline in growth rate and rapid entry into stationary phase. The proteomes for these two temperatures were also markedly different to cells cultured at lower temperatures and to each other, while sharing many common trends with the global responses documented for LAB, particularly in greater abundance of ABC- and PTS-transporters plus rerouting carbohydrate metabolism. Many of the proteins with highly altered abundance, or unique detection, at 45°C were functionally uncharacterized but domain structures detected and phylogenetic analyses infer presumptive activity of proteins which have not previously been documented in heat stress responses in lactobacilli.

Chaperones GroES, GroEL, DnaK and GrpE were up-regulated significantly only at 45°C, consistent with many previous reports for heat shock responses in LAB, while DnaJ was increasingly more abundant with temperature. Although the gene cluster including the *hrcA* heat inducible regulator, *grpE*, *dnaK* and *dnaJ* are considered to form an operon which is conserved across *Lactobacillus* species (De Angelis *et al*. 2004), our proteomic data indicated separate regulation of these genes as growth temperature increased, an observation supported by detecting promoters in intergenic regions, which has not been reported for heat shock responses in LAB. Expression of the chaperone systems during prolonged heat stress was more enhanced than the proteases, suggesting that protein refolding and recycling were preferred to proteolysis as a mechanism for protein quality control in *L. casei* GCRL163. A novel observation from our data was the consistent increase in abundance with temperature of an α-crystallin domain protein encoded by gene locus BN194_29440 and annotated as an acid

shock protein (Consortium 2016), was more highly up-regulated in response to heat stress compared to all other molecular chaperones. The α-crystallin heat shock proteins possess a conserved central α-crystallin domain region of about 80 amino acids and form part of the highly synergistic multi-chaperone network induced usually under stress conditions and weakly under normal conditions in cells (Narberhaus 2002). The ATP-independent chaperone activity is limited to the prevention of irreversible protein aggregation by binding to unfolding intermediates and may pay a role in cell surface integrity (Narberhaus 2002). The expression of the α-crystallin heat shock protein family is restricted to some members of the eukaryotes, archaea and bacteria, and is absent, for example, in *Mycoplasma genitaliun* (Fraser *et al*. 1995) and *Helicobacter pylori* (Tomb *et al*. 1997). In LAB, including *L. delbrueckii*, *L. helveticus*, *Oenococcus oeni* and *S. thermophilus*, small heat shock proteins related to eukaryotic α-crystallins have been reported (Jobin *et al*. 1998; Weidmann *et al*. 2017). Interestingly, despite the extensive literature on the mechanism of the highly-conserved chaperone proteins in protein folding in numerous bacterial species, Papadimitriou *et al*. (2016) noted that studies on the mechanisms of action of chaperones in LAB are lacking (Papadimitriou *et al*. 2016). This suggests that there is scope for further investigations on the role of small HSPs, particularly in context of the greater expression of the Hsp20 protein as culture temperature increased (reported in the current study) and prior reports on stress-induced small HSPs in *L. plantarum* and *O. oeni* (Papadimitriou *et al*. 2016).

Our current investigation further provides findings on the involvement of the chaperone systems in the post-translational secretion in *L. casei* GCRL163 during prolonged heat stress, with the BN194_10000 protein, consisting of 136 amino acids and with a superfamily domain structure consistent with a protein export chaperone SecB, identified. The translocation of proteins across the membrane is thought to be facilitated by the

chaperone function of the heat shock proteins in bacteria and eukaryotes (Hannavy *et al*. 1993; Phillips & Silhavy 1990). In *E. coli*, secretion of outer membrane proteins occurs principally through the SecB-dependent pathway with SecB binding to non-native proteins and presenting them in competent state to SecA in the Sec translocon (Chatzi *et al*. 2013; Kumamoto 1989). However, in SecB deficient strains, DnaK, DnaJ, GroES-EL and GrpE are the main chaperones (Phillips & Silhavy 1990; Wild *et al*. 1996). SecB is essential for protein folding in *E. coli* at low temperatures and was shown to compete with the GroES-EL and DnaK-DnaJ-GrpE chaperones for unfolded proteins: as growth temperature increases, SecB is repressed while chaperones are induced (Ullers *et al*. 2007). Few SecB homologs in Gram-positive bacteria have been linked experimentally with preventing protein folding and aggregation (Schneewind & Missiakas 2012; Scott & Barnett 2006) and most of the current knowledge concerning the Sec pathway is based on studies in *E. coli* and other Gram-negative species. The exceptions are the CsaA protein in *B. subtilis* (110 amino acids) (Müller *et al*. 1992) and the SecB-like protein of *Mycobacterium tuberculosis* (Bordes *et al*. 2011) (181 amino acids) (Müller *et al*. 1992), which are not structurally similar to the Gram-negative SecB proteins but are able to complement *E. coli* SecB mutants (Müller *et al*. 1992; Müller *et al*. 2000). A phylogenetic analysis of 3,813 SecB genes, identified in UniProt in bacteria with full taxonomic lineage and across all phyla, showed that the sequence similarity between genes was often very low but the protein architecture is preserved despite differences in protein size (mean of 157 ±27 amino acids) (Yan & Wu 2015). The modelled 3D-structures of *L. casei* GCRL163 and *L. rhamnosus* SecB homolog proteins showed architecture generally similar to the SecB of *E. coli*, and close structural alignment between the proteins was evident. However, this structure differed from the *E.coli, L. plantarum* and *B. licheniformis* model structures, notably the absence of the α2, C-terminal helix. Although the structure of the SecB protein is important for forming a tetramer which is a ‘dimer of

dimers' (Huang *et al*. 2016), prior studies had shown that deletion of the α2-helix did not alter client protein binding but did debilitate interactions with SecA (Zhou & Xu 2005). It remains to be demonstrated whether the arrangement of residues in the presumptive *L. casei* SecB protein supports dimer plus tetramer formation and whether the structure supports client protein binding. Evidence for the proposed function of the SecB homolog in *L. casei* in the current study is based on global architectural similarity and the observed expression pattern of the protein during culture at temperatures below optimum. A reasonable first step in demonstrating functionality would be determining whether expression of the *L. casei secB* complements knock-out mutants of *E. coli*, similar to the studies which demonstrated the chaperone role of CsaA in *B. subtilis*. To our knowledge, this is the first report of a SecB homolog in lactobacilli, which are generally thought to lack SecB (Kleerebezem *et al*. 2010).

In bacteria, preprotein with N-terminal domain, hydrophobic core and C-terminal domain can be translocated through co-translational system by binding SRP (signal recognition particle) formed by Ffh protein (Mori & Ito 2001). Although the abundance of Ffh was not modulated by the heat stress, several components of the Sec secretion pathway (SecA, which channels the preprotein to SecYEG and translocase subunit SecY), proteins YajC and LepB (responsible for the release of preprotein and matured protein from SecYEG channel) and PrsA were more abundant at 40 and 45°C. Greater abundance of these proteins suggests that greater levels of protein secretion to or through the cell surface may occur during prolonged heat stress. Despite the importance of the surface and extracellular proteome of lactobacilli in probiotic function, there is relatively little reported on the impact of stress on the secretome (Kleerebezem *et al*. 2010) with the exception of recent studies on S-layer producing species (Klotz *et al*. 2017). Examining the surface and secreted proteins

following culture at supra-optimal temperatures of *L. casei* GCRL163 will expand our knowledge on the yet uncharacterized heat stress responses of lactobacilli.

As observed for other stress responses in LAB (Papadimitriou *et al*. 2016; De Angelis *et al* 2016), the expression of proteins around pyruvate metabolism was impacted by growth at 40 and 45°C. However, the pattern of expression differed at the two temperatures: at 40°C, the anticipated upregulation of proteins associated with pyruvate conversion to acetyl-CoA thence acetate was observed (Papadimitriou *et al*. 2016), whereas at 45°C, the abundance of the Pdh complex subunits involved in the formation of acetyl-CoA was similarly enhanced while all other proteins associated with formation of acetate, with the exception of Pta, were repressed. A key difference at 45°C was the large increase in abundance of AdhE, which has not been reported previously for *L. casei* or other LAB. The fate of acetyl-CoA was not experimentally explored in this study, but the pattern of protein expression suggests altered carbon flux to acetate with possible accumulation of acetyl-P or formation of acetaldehyde or ethanol end products and lowered energy production through conversion of acetyl-P to acetate at 45°C. In acid stress, pyruvate metabolism is rerouted into the synthesis of fatty acid synthesis in *L. delbrueckii* subsp. *bulgaricus* and *L. rhamnosus* (Koponen *et al*. 2012; Zhai *et al*. 2014), a route which is not taken in prolonged heat stress given the decline in expression of fatty acid synthesis proteins observed at 45°C. Furthermore, all of the ATP synthase subunits were up-regulated at 40°C and more so at 45°C. These proteins are linked to the maintenance of intracellular pH through proton extrusion by the F_0F_1-ATPase and maintenance of a PMF by the membrane bound F_0F_1-ATP (Konings *et al*. 1997; Cotter & Hill 2003). Our data demonstrate that under prolonged heat stress cellular energy is sourced more through ATP synthase activity in this strain.

Modulation of some glycolytic pathway proteins is an important mechanism in LAB for generating energy in the form of ATP during heat and other stresses (Di Cagno *et al*. 2006; Hahne *et al*. 2010; Wilkins *et al*. 2001), although the patterns of modulation vary with genera and stress type (Papadimitriou *et al*. 2016). It can be inferred that *L. casei* GCRL163 cells consume more energy at elevated temperature, which is consistent with ATP usage by chaperones at higher temperature (Rothman & Schekman 2011). The bacteria therefore establish a network of mechanisms to generate energy for survival during thermal stress but are very conservative in the energy-dispensing processes thereby shutting down high energy-requiring pathways, such as fatty acid biosynthesis, especially at 45°C. Our data suggested that oleic acid was the principal unsaturated fatty acid at this temperature with vaccenic acid greatly reduced, at all growth phases. At other temperatures, oleic acid decreased from exponential to stationary phase. This temperature-dependent reduction in the degree of fatty acid unsaturation was also observed in *L. fermentum* as a result of temperature-dependent shift between vaccenic to oleic acid below 20°C and above 26°C with oleic acid cyclized to cyclopropane acids by cyclopropane synthetase (Suutari & Laakso 1992) The synthesis of vaccenic and oleic acids is not well defined under anaerobic conditions although it is hypothesised this occurs by insertion of a double bond during chain elongation by the action of the Fab enzymes (Suutari & Laakso 1992; Mansilla *et al*. 2004). Decrease in vaccenic acid production may indicate either temperature-sensitive impairment of insertion of a double bond during chain elongation (together with reduction of fatty acid synthesis enzymes) or failure to subsequently epimerise oleic into vaccenic acid. This remains a subject for further investigation.

In *L. casei* GCRL163, expression of cyclopropane fatty acid acyl synthase which could be responsible for the cyclization of oleic acid to cyclopropane was up-regulated

maximally at 35°C with only minor increases at 40°C and 45°C relative to 30°C. Our GC/MS data also revealed novel cyclopentenyl moieties produced in all the growth phases which might have been synthesized from the cyclization of oleic acid with decreased synthesis at 45°C. Cyclopentenyl fatty acids have previously been reported in plants (Bandi & Mangold 1969) and oil (Oliveira *et al*. 2009) but not in bacteria. To the best of our knowledge this represents the first report of the cyclopentenyl moieties in LAB. Fatty acid biosynthesis is an energy intensive process and cells have the capacity to adjust existing fatty acids thereby conserving energy that would be otherwise spent in synthesising new fatty acids (Jerga & Rock 2009), which we suggest occurs during growth at 45°C.

Several other proteins related to DNA repair and recombination, post-translational modification, amino acid metabolism, nucleic acid and nucleotide metabolism were also repressed at higher growth temperatures. Indeed, the pattern of protein expression for cells cultured at 45°C (repression of DnaA, ribosome content and proteins associated with purine biosynthesis) is reminiscent of transition into stationary phase (Cohen *et al*. 2006; Laakso *et al*. 2011), which is consistent with the growth kinetics observed. However, greater abundance of proteins involved in sugar uptake, the tagatose-phosphate pathway (Lac proteins) and greater expression of an α-L-fucosidase at 45°C, plus upregulation of proteins involved in forming fructose-6-phosphate (ManD, NagA), suggest both carbon source scavenging and possible turnover of peptidoglycan. The unique detection of Asp23_2 at 45°C may also indicate cell surface structural changes beyond membrane lipid content, given the documented role of this class of stress protein in cell wall stress in *Staphylococcus aureus* (Müller *et al*. 2014) and the recent implication of this protein in cell surface changes in gentamycin resistant *L. casei* (Zhang *et al*. 2018). Our study of proteomic changes during growth at inhibitory, supra-optimal temperatures has revealed new findings on how *L. casei*

GCRL163 responds to prolonged heat stress and opens up further avenues for investigation on the network of physiological changes required to enhance cell survival.

In order to gain further in-depth insight into how *L. casei* GCRL163 modulated cell surface proteins under prolonged heat stress and the impact of heat stress on the cell adhesive property, we used different approaches to extract the cell surface proteins and performed cell adhesion assays as described in Chapter 4.

3.5 Figures and Tables

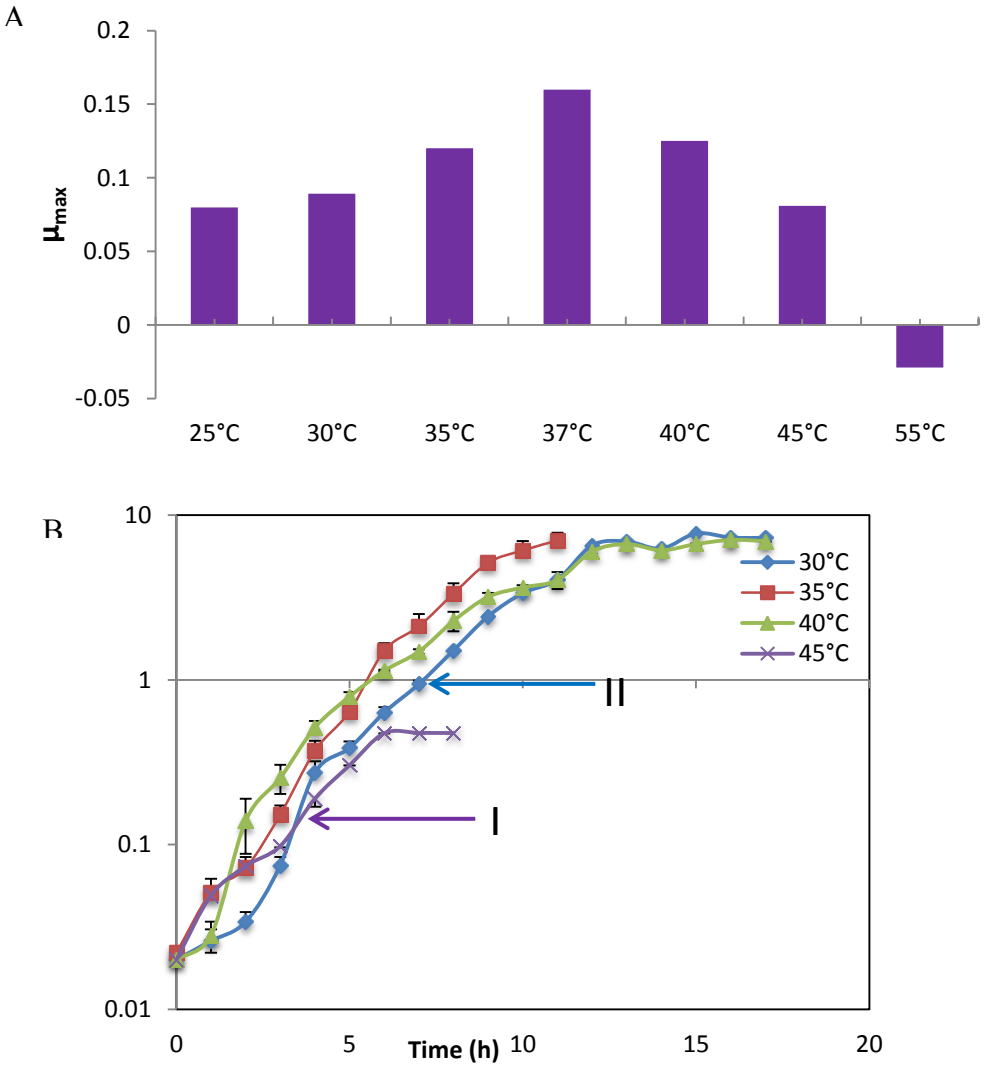


Figure 3. 1. Growth rates of *L. casei* GCRL163 at different temperatures. (A) Maximum specific growth rates of *L. casei* GCRL163 in MRS broth at different temperatures. (B) Growth curves of *L. casei* GCRL163 at 30°C, 35°C, 40°C and 45°C. Arrow I and II represent the points of harvest at mid-exponential phase for 45°C and other temperatures (30°C, 35°C and 40°C) respectively. Bacterial cells for proteomic studies were harvested at mid-log [7 h (30°C), 6 h (35°C), 5 h (40°C) and 4 h (45°C)] corresponding to OD_{600} 1.04, 1.50, 0.69 and 0.19 respectively while cell samples for fatty acid compositional profiles were harvested at mid-exponential, late-exponential and stationary phases. Preliminary experiments indicated growth at 37°C as optimal temperature and no significant growth was observed beyond 50°C.

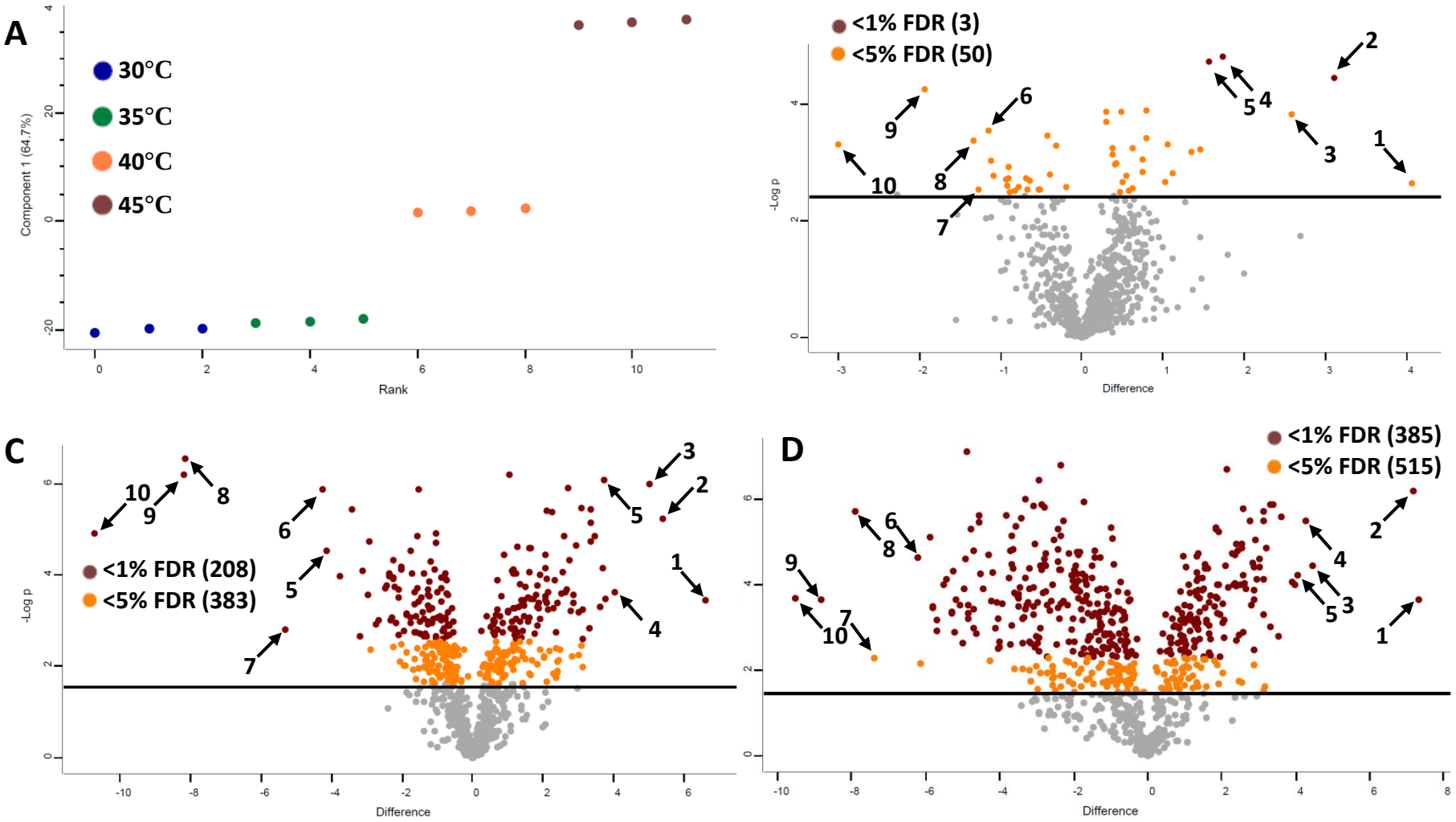

A
30°C
35°C
40°C
45°C
Component 1 (64.7%)
Rank
<1% FDR (3)
<5% FDR (50)
-Log p
Difference
C
<1% FDR (208)
<5% FDR (383)
-Log p
Difference
D
<1% FDR (385)
<5% FDR (515)
Difference

Figure 3. 2. Dot plot representation of the impact of prolonged heat exposure on the proteome of *L. casei* GCRL163. (A) Principal Component Analysis (B) two-sided *t*-test analysis of the protein LFQ expression data for the temperature comparisons of (I) 35°C *vs* 30°C, (II) 40°C *vs* 30°C and (III) 45°C *vs* 30°C. The numbers of significant differentially-expressed proteins at 1% and 5% FDR thresholds after correction for multiple hypothesis testing (brown dots and orange dots, respectively) are shown in parentheses. Significant proteins with the highest fold-difference between 30°C LFQ values and 35°C, 40°C and 45°C LFQ values, respectively, are indicated by arrows and the corresponding protein identities and $\log_2$ fold-changes are shown in Table 3.1.

Table 3. 1. Most highly differentially altered proteins in *L. casei* GCRL163, following culture at different temperatures as annotated on the Volcano plots in Figure 3. 2B-D.

#	Protein name	Protein ID	Peptides	MS/MS	*p*_35/30°C	FDR	Log2_35/30°C	Protein name*
1	**ThiM**	K0N1Q6	6	124	0.0022575	<5%	4.06	Hydroxyethylthiazole kinase
2	**BN194_29440**	K0MYM2	9	70	3.50E-05	<1%	3.10	Uncharacterized protein /Acid shock protein, alpha crystalline, Hsp20 domain (IPR002068)*
3	**BN194_05550**	K0N257	2	11	0.0001492	<5%	2.58	Uncharacterized protein
4	**DnaJ**	K0N5J4	13	83	1.54E-05	<1%	1.74	Chaperone protein DnaJ
5	**MetE**	K0N658	24	121	1.87E-05	<1%	1.56	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
6	**GlnA_2**	K0MW64	28	474	0.0002813	<5%	-1.15	Glutamine synthetase
7	**PepC**	K0NC50	14	37	0.0028795	<5%	-1.27	PepC protein
8	**Sph**	K0N5Q0	9	32	0.000424	<5%	-1.33	Sph protein
9	**Cap4C**	K0N7E3	22	220	5.47E-05	<5%	-1.94	UTP--glucose-1-phosphate uridylyltransferase
10	**PepT**	K0N213	11	33	0.0004884	<5%	-3.00	Peptidase T

#	Protein name	Protein ID	Peptides	MS/MS	*p*_40/30°C	FDR	Log2_40/30°C	Protein name
1	**ThiM**	K0N1Q6	6	124	0.0003666	<1%	6.61	Hydroxyethylthiazole kinase
2	**BN194_29440**	K0MYM2	9	70	6.01E-06	<1%	5.39	Uncharacterized protein /Acid shock protein, alpha crystalline, Hsp20 domain (IPR002068)*
3	**DppE_3**	K0NAD1	13	33	1.02E-06	<1%	5.03	DppE_3 protein
4	**BN194_19800**	K0N670	4	12	0.0002315	<1%	4.05	Uncharacterized protein /ABC transporter substrate-binding protein (IPR007487)*
5	**MetQ_2**	K0MV23	8	48	0.0003446	<1%	3.78	Lipoprotein
6	**PepC**	K0NC50	14	37	1.34E-06	<1%	-4.24	PepC protein
7	**PurK_2**	K0NB96	9	78	0.0001214	<1%	-5.31	N5-carboxyaminoimidazole ribonucleotide synthase
8	**PurD**	K0MWC4	16	177	0.0002099	<1%	-8.14	Phosphoribosylamine--glycine ligase
9	**PurH**	K0N634	18	286	1.89E-06	<1%	-8.18	Bifunctional purine biosynthesis protein PurH
10	**PurC**	K0N5S8	12	145	0.0002252	<1%	-10.73	Phosphoribosylaminoimidazole-succinocarboxamide synthase

#	Protein name	Protein ID	Peptides	MS/MS	*p*_45/30°C	FDR	Log2_45/30°C	Protein name
1	**ThiM**	K0N1Q6	6	124	0.0002278	<1%	7.33	Hydroxyethylthiazole kinase
2	**BN194_29440**	K0MYM2	9	70	6.34E-07	<1%	7.17	Uncharacterized protein /Acid shock protein, alpha crystalline, Hsp20 domain (IPR002068)*
3	**BN194_30140**	K0MYT6	5	14	3.49E-05	<1%	4.44	Uncharacterized protein /Aldolase-type TIM barrel (IPR013785)
4	**FruA_3**	K0N4S1	10	78	3.23E-06	<1%	4.26	FruA_3 protein
5	**BN194_30000**	K0N979	7	21	5.77E-05	<1%	4.07	Uncharacterized protein /Glycosyl transferase family 1 (IPR001296)*
6	**PepC**	K0NC50	14	37	2.26E-05	<1%	-6.19	PepC protein
7	**OppA_2**	K0MWL6	19	174	0.0051703	<5%	-7.39	OppA_2 protein
8	**PurH**	K0N634	18	286	1.89E-06	<1%	-7.90	Bifunctional purine biosynthesis protein PurH
9	**PurC**	K0N5S8	12	145	0.0002252	<1%	-8.83	Phosphoribosylaminoimidazole-succinocarboxamide synthase
10	**PurD**	K0MWC4	16	177	0.0002099	<1%	-9.53	Phosphoribosylamine--glycine ligase

*UniProt submitted or reviewed name for *L. casei* W56 proteins; domains which indicate potential function are specified in parentheses.

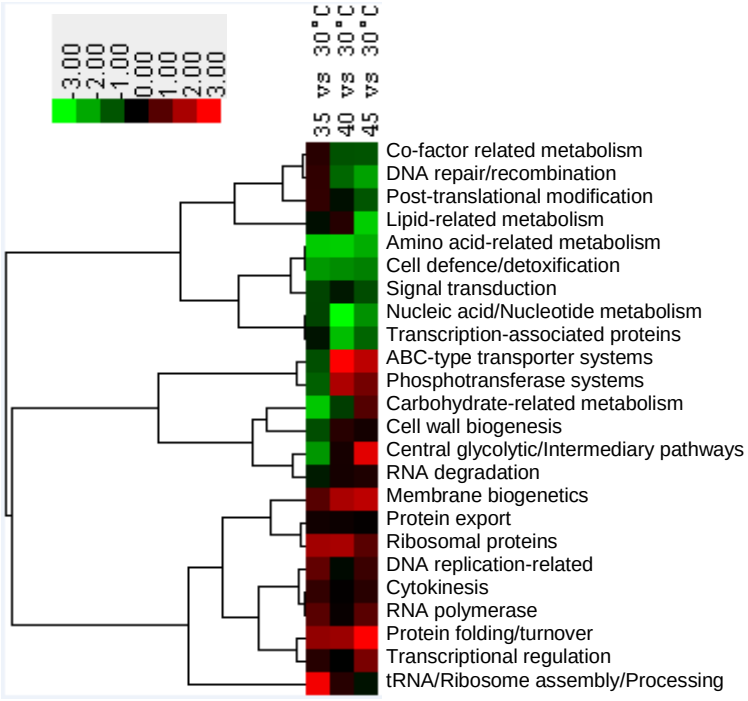


Figure 3. 1. Heat map depicting change in abundance trends in functional groups of proteins in *L. casei* GCRL163 based on *t*-test values derived from T-profiler analysis at different temperatures (35°C, 40°C, 45°C compared to 30°C) using cluster v.3.0 software. The overall proteomic data at different temperatures (top) were compared to the protein functional groups (left dendogram) using unsupervised hierarchical clustering on the basis of uncentered correlation. Analysis of the proteins based on the functional classification as indicated by the colour scale based on *t*-values revealed differentially expressed proteins.

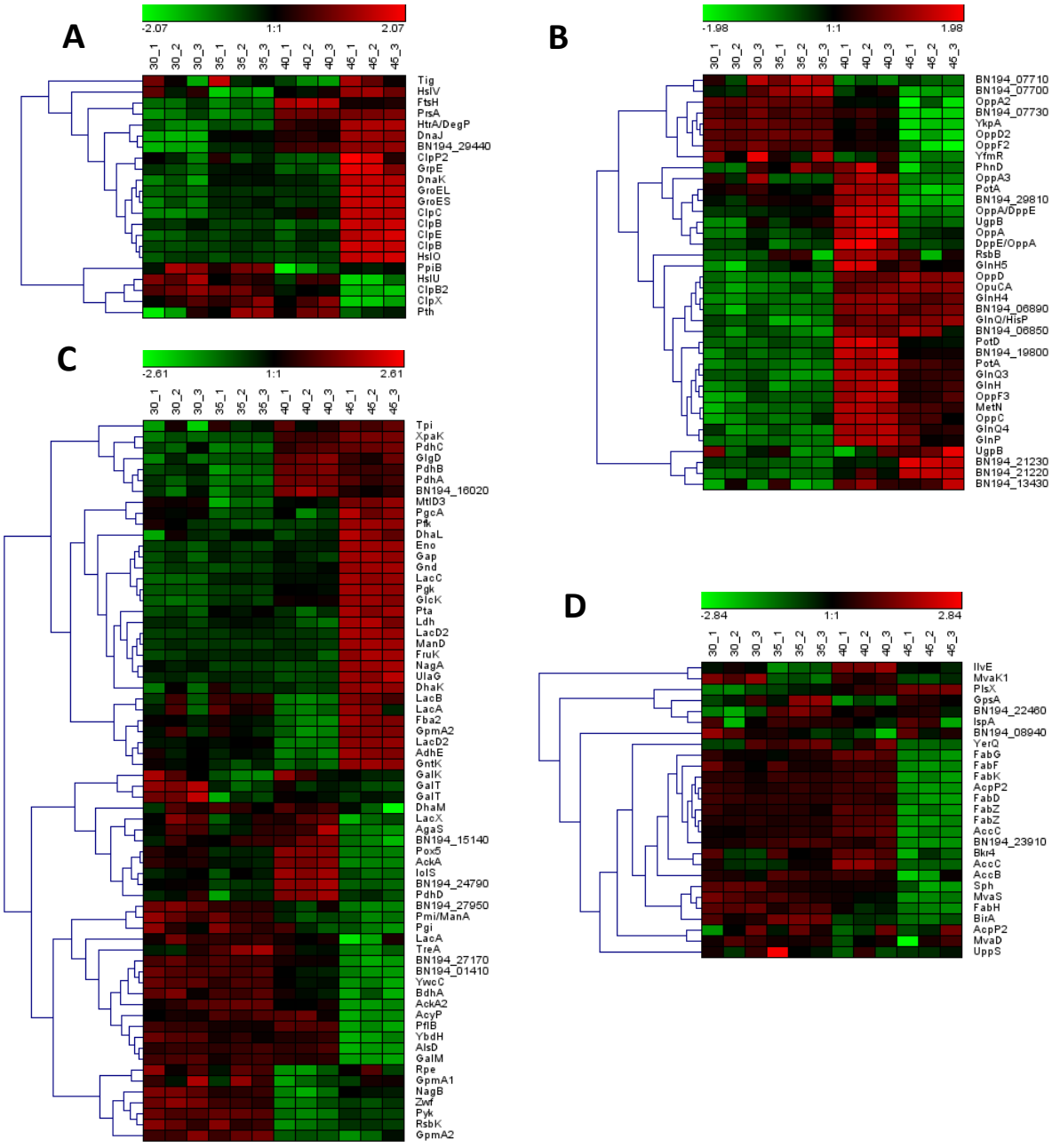


Figure 3. 2. Hierarchical cluster analysis of the abundance of selected proteins in functional classes detected in *L. casei* GCRL163 CFEs at different temperatures. Mean-centred (Z-scored) LFQ data is shown for triplicates for (A) Protein folding and turnover (B) ABC-type transporter system components (C) Glycolytic/intermediary and carbohydrate metabolism and D) and Fatty acid metabolism functional classes.

Table 3. 2. Proteins not detected in CFEs of *L. casei* GCRL163 for one or more growth temperatures. Proteins were scored as detected (+) when present in all three technical replicates at a specific temperature, or absent (-) when below the level of detection in all three replicates.

Gene name	Protein ID	Gene locus	30°C	35°C	40°C	45°C	Protein names*	Protein functions
thiM	K0N1Q6	BN194_03200	-	+	+	+	Hydroxyethylthiazole kinase	Thiamine biosynthesis
rihC	K0N803	BN194_03870	-	-	+	+	Non-specific ribonucleoside hydrolase	RNA metabolism, nucleotide salvaging
	K0N8Q2/K0N7Z0	BN194_27750/ BN194_27760	-	(+)**	+	+	Putative Alpha-L-fucosidase	Fucose metabolism?
levE_4	K0ND69	BN194_27770	-	-	+	+	Fructose-specific phosphotransferase enzyme IIB component	Sugar uptake
	K0MV08	BN194_13440	-	-	+	+	Uncharacterized ABC transporter ATP-binding protein Mb1303c	Lipid export?
asp23_2	K0N1F0	BN194_01950	-	-	-	+	Alkaline shock protein 23 family protein	General stress protein
metN	K0N4L1	BN194_13750	-	-	+	-	Methionine import ATP-binding protein MetN	Methionine uptake
	K0N6W6	BN194_23110	-	-	+	-	ABC transporter	Toxic substance efflux?
ppdK	K0NAC6	BN194_24730	(+)	-	+	-	Pyruvate, phosphate dikinase	Glycolysis
	K0NB06	BN194_27880	-	-	+	-	Autoinducer-2 (AI-2) kinase/class II aldolase	Fucose metabolism?
tagT	K0ND99	BN194_27920	-	-	+	-	PTS IIA-like nitrogen-regulatory protein	Transport, galactitol uptake?
	K0N181	BN194_01150	+	+	+	-	Uncharacterized protein	Unknown
	K0N7D4	BN194_01870	+	+	+	-	Uncharacterized protein	Unknown
	K0N5D9	BN194_03130	+	+	+	-	Transcriptional regulator TetR family with acetyltransferase GNAT family domain	Regulation
	K0N281	BN194_04410	+	+	+	-	Uncharacterized protein MJ1651, S-adenosyl-l-methionine hydroxide adenosyltransferase, N- and C-termini	Unknown
Sph	K0N5Q0	BN194_04930	+	+	+	-	Oleate hydratase	Fatty acid degradation or detoxification?
mutS2	K0N359	BN194_08560	+	+	+	-	Endonuclease MutS2	DNA repair (mismatch)
Ung	K0N758	BN194_11330	+	+	+	-	Uracil-DNA glycosylase	DNA repair (base excision)
yerH	K0MUH2	BN194_11940	+	+	+	-	Sex pheromone lipoprotein, CamS superfamily	Unknown

yfmL	K0NA13	BN194_12120	+	+	+	-	Putative ATP-dependent RNA helicase yfmL	tRNA/Ribosome assembly/processing
ycnE	K0N7R3	BN194_13230	+	+	+	-	Putative monooxygenase, YcnE protein	Unknown
pepD	K0N7S0	BN194_13380	+	+	+	-	Probable dipeptidase	Peptidase for amino acid acquisition
ytsP	K0N4Q9	BN194_14450	+	+	+	-	YtsP protein	Unknown signal transduction
ysoA	K0N4Y3	BN194_15300	+	+	+	-	Uncharacterized protein	Unknown
hutG	K0N5I0	BN194_17350	+	+	+	-	N-formylglutamate amidohydrolase	Histidine metabolism
	K0NAY8	BN194_17420	+	+	+	-	Uncharacterized protein, ASCH/PUA domain	Unknown, RNA processing?
Smc	K0N5H9	BN194_17860	+	+	+	-	Chromosome partition protein Smc	Cell division (chromosomal segegration)
Fmt	K0N5J2	BN194_18060	+	+	+	-	Methionyl-tRNA formyltransferase	Protein synthesis; translation initiation
xseA	K0N5U4	BN194_18200	+	+	+	-	Exodeoxyribonuclease 7 large subunit	DNA repair (mismatch)
yhaM	K0NB84	BN194_19070	+	+	+	-	3'-5' Exoribonuclease yhaM	RNA degradation (mRNA turnover?)
	K0N5U0	BN194_19510	+	+	+	-	GNAT family acetyltransferase	Unknown
	K0N5Z4	BN194_20160	+	+	+	-	Uncharacterized protein	Unknown
oppF_2	K0MWL1	BN194_20590	+	+	+	-	Oligopeptide transport ATP-binding protein OppF	Oligopeptide uptake
oppD_2	K0N6C6	BN194_20600	+	+	+	-	Oligopeptide transport ATP-binding protein OppD	Oligopeptide uptake
accC	K0N6M5	BN194_22510	+	+	+	-	Biotin carboxylase	Fatty acid biosynthesis
fabD	K0NBT1	BN194_22570	+	+	+	-	Malonyl CoA-acyl carrier protein, Transacylase	fatty acid biosynthesis
	K0N7J8	BN194_23650	+	+	+	-	Amino acid-related metabolism	Unknown
dnaX	K0MXD3	BN194_23990	+	+	+	-	DNA polymerase III subunit gamma/tau	DNA replication, elongation
	K0N7R2	BN194_24650	+	+	+	-	YjdJ protein, GNAT family acetyltransferase	Unknown
pepC	K0NC50	BN194_24670	+	+	+	-	Aminopeptidase C	Peptidase for amino acid acquistion
yqiG	K0NAG4	BN194_25330	+	+	+	-	Probable NADH-dependent flavin oxidoreductase	Unknown
	K0N7Q6	BN194_27210	+	+	+	-	Uncharacterized protein	Unknown
	K0NAX5	BN194_27530	+	+	+	-	Uncharacterized protein	Unknown
	K0MT25	BN194_06540	+	+	-	-	YieF_2 protein, Oxidoreductase activity	Unknown
purC	K0N5S8	BN194_19360	+	+	(+)	-	Phosphoribosylaminoimidazole-succinocarboxamide synthase	Purine biosynthesis
purL	K0N9H6	BN194_19330	+	+	-	-	Phosphoribosylformylglycinamidine synthase subunit PurL (FGAM synthase) (EC 6.3.5.3)	Purine biosynthesis
purM	K0NB95	BN194_19320	+	+	-	-	Phosphoribosylformylglycinam	Purine biosynthesis

							idine cyclo-ligase (EC 6.3.3.1)	
purQ	K0MWC7	BN194_19340	+	+	-	-	Phosphoribosylformylglycinamidine synthase subunit PurQ (FGAM synthase) (EC 6.3.5.3)	Purine biosynthesis

* Protein names from UniProt reviewed or submitted names for *L. casei* W56 or from the RAST SEED finder annotation of *L. casei* GCRL163 genome (Nahar *et al.* 2017), with validation by BLASTP in KEGG and NCBI

** Denotes very low LFQ intensity (log_2 values <<20)

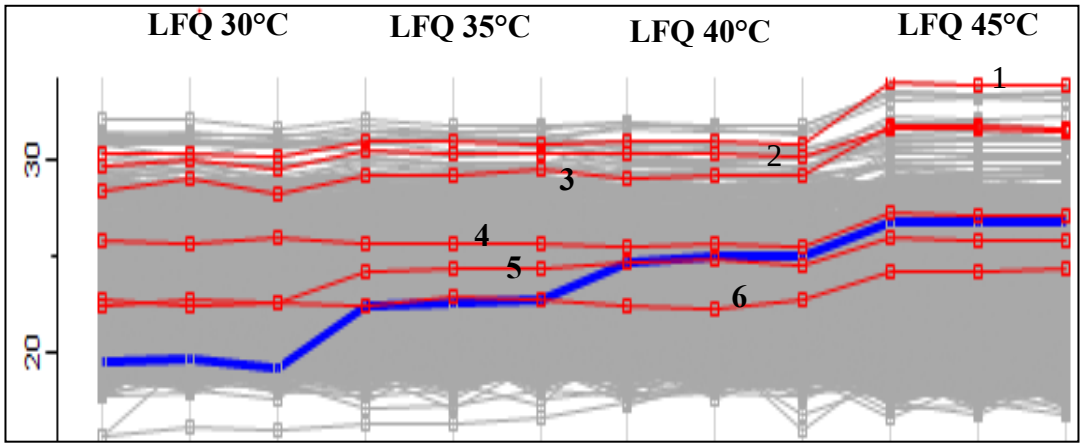


Figure 3. 5. Abundance of selected chaperone proteins in triplicate samples of CFEs for cells harvested at mid-exponential growth at temperatures from 30 to 45°C. LFQ data is $\log_2$ transformed (Y-axis). 1, GroEL; 2, GroES; 3, DnaK; 4, ClpB; 5, DnaJ; 6, hsp33 and 7, BN194_29440. Note that BN194_29440 (which contains an α-crystallin domain of the Hsp20 family chaperones) is more highly abundant as the temperature increases, whereas the other molecular chaperones are elevated primarily at 45°C.

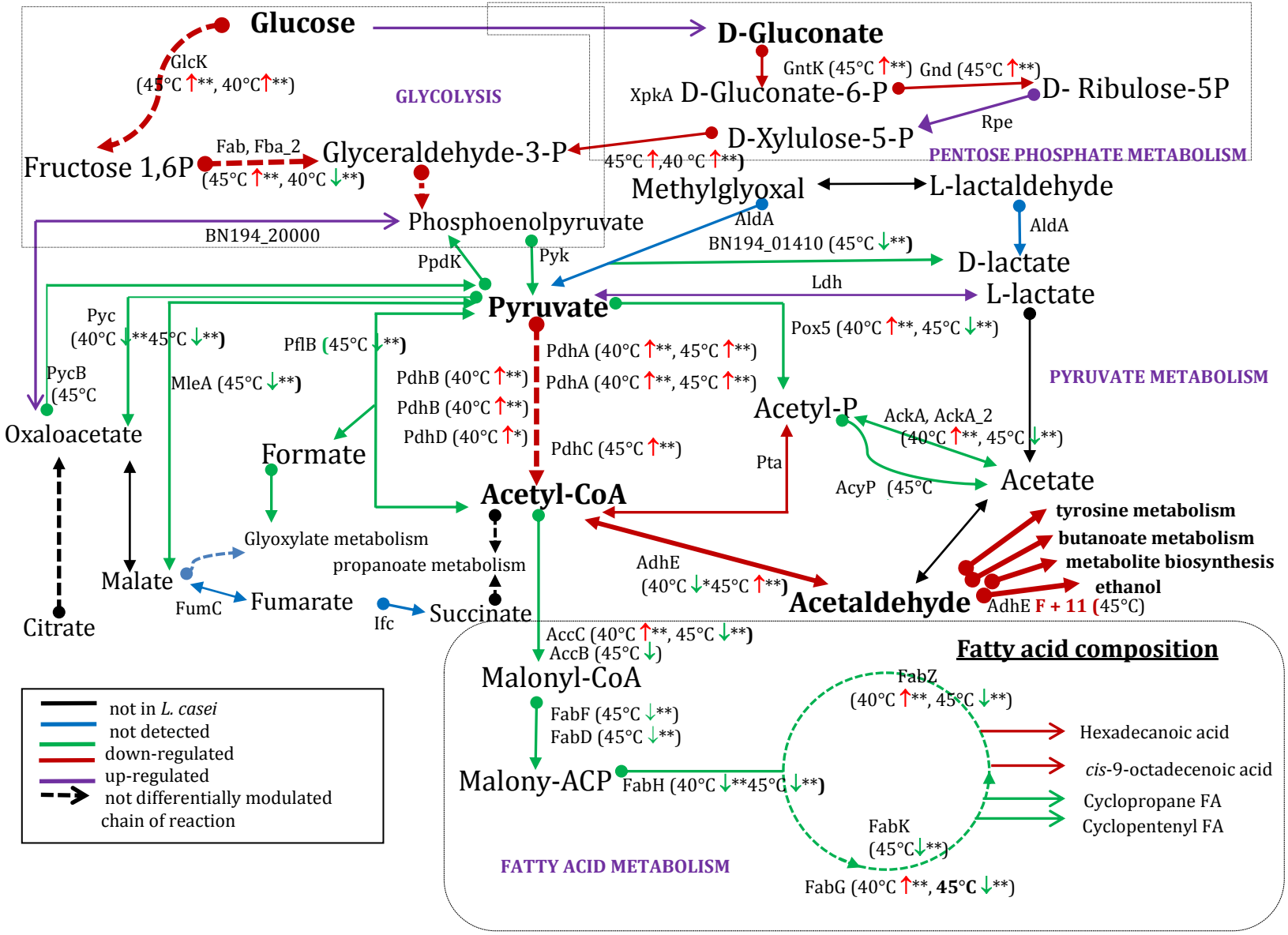

Glucose
D-Gluconate
GlcK
(45°C ↑**, 40°C↑**)
GntK (45°C ↑**) Gnd (45°C ↑**)
XpkA D-Gluconate-6-P
D- Ribulose-5P
Rpe
GLYCOLYSIS
D-Xylulose-5-P
PENTOSE PHOSPHATE METABOLISM
Fab, Fba_2
(45°C ↑**, 40°C ↓**)
Fructose 1,6P
Glyceraldehyde-3-P
45°C ↑,40 °C ↑**)
Methylglyoxal
L-lactaldehyde
AldA
AldA
BN194_20000
Phosphoenolpyruvate
BN194_01410 (45°C ↓**)
D-lactate
PpdK
Pyk
Ldh
L-lactate
Pyruvate
Pyc
(40°C ↓**45°C ↓**)
PflB (45°C ↓**)
Pox5 (40°C ↑**, 45°C ↓**)
PycB
(45°C
MleA (45°C ↓**)
PdhA (40°C ↑**, 45°C ↑**)
PdhB (40°C ↑**)
PdhA (40°C ↑**, 45°C ↑**)
PYRUVATE METABOLISM
Acetyl-P
PdhB (40°C ↑**)
PdhD (40°C ↑*)
PdhC (45°C ↑**)
AckA, AckA_2
(40°C ↑**, 45°C ↓**)
Oxaloacetate
Formate
Pta
AcyP (45°C
Acetate
Acetyl-CoA
tyrosine metabolism
butanoate metabolism
metabolite biosynthesis
ethanol
Glyoxylate metabolism
propanoate metabolism
AdhE
(40°C ↓*45°C ↑**)
Malate
Acetaldehyde
AdhE F + 11 (45°C)
FumC
Fumarate
Ifc
Succinate
Citrate
AccC (40°C ↑**, 45°C ↓**)
AccB (45°C ↓)
Fatty acid composition
Malonyl-CoA
FabZ
(40°C ↑**, 45°C ↓**)
FabF (45°C ↓**)
FabD (45°C ↓**)
Hexadecanoic acid
cis-9-octadecenoic acid
Malony-ACP
FabH (40°C ↓**45°C ↓**)
Cyclopropane FA
Cyclopentenyl FA
FabK
(45°C↓**)
FATTY ACID METABOLISM
FabG (40°C ↑**, 45°C ↓**)
not in L. casei
not detected
down-regulated
up-regulated
not differentially modulated
chain of reaction

Figure 3. 6. Schematic metabolic map adapted from KEGG pathways showing changes in abundance of some significant proteins in glycolysis, the pentose phosphate pathway, the Pyruvate and fatty acid metabolism at 40°C and 45°C in relation to 30°C. The annotated pathways show changes at growth temperature 45°C. Coloured arrows represent up- (red) or downregulation (green) or not significantly modulated (purple) or not detected (blue) and not in known to be present in *L. casei* (black). Dashed-lines represent a series of reactions, where every enzyme or substrate not shown. Up-regulated proteins are indicated by red and down-regulated by green arrows with asterisk indicating FDR (**, FDR<1% and *, FDR<5%). Glck, Glucokinase; Fba, Fructose-bisphosphate aldolase; PfkA, 6-phosphofructokinase; Ldh, Lactate dehydrogenase; Pyc, Pyruvate carboxylase; PycB, Oxaloacetate decarboxylase, alpha subunit; MleA, Malate dehydrogenase; PflB, Formate acetyltransferase; PdhABCD, Pyruvate dehydrogenase complex; PoX5, Pyruvate oxidase; AdhE, Aldehyde-alcohol dehydrogenase; Ack, Acetate kinase; AccBC, Acetyl-CoA carboxylase carboxyl transferase complex; FabDFGHK, 3-oxoacyl-ACP synthase complex; Pta, Phosphate acetyltransferase; AcyP, Acylphosphatase; Gnd, 6-phosphogluconate dehydrogenase; XpkA, Xylulose-5-phosphate phosphoketolase; GntK, Gluconokinase and PRPP, phosphor-alpha-D-ribose 1-diphosphate.

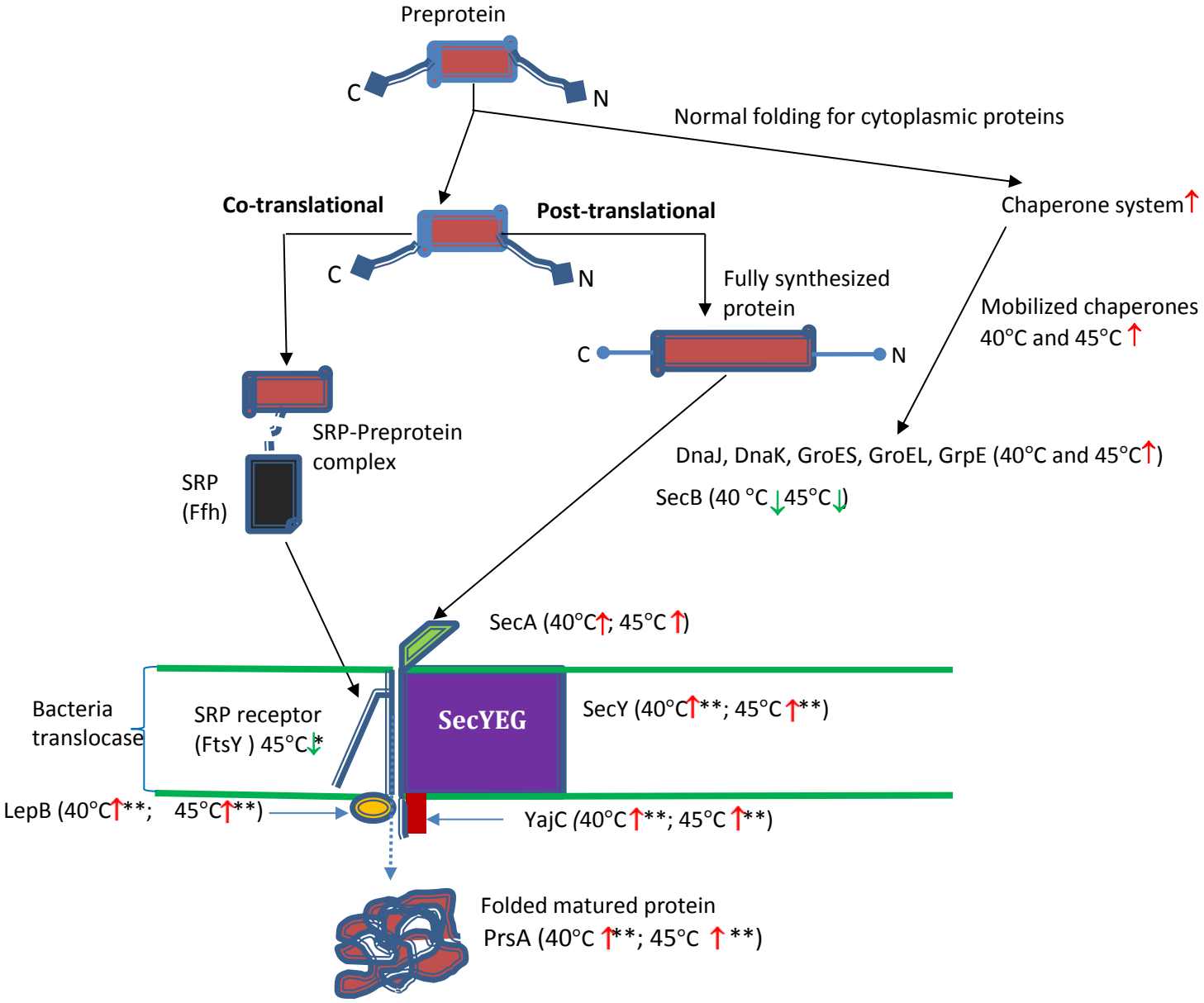
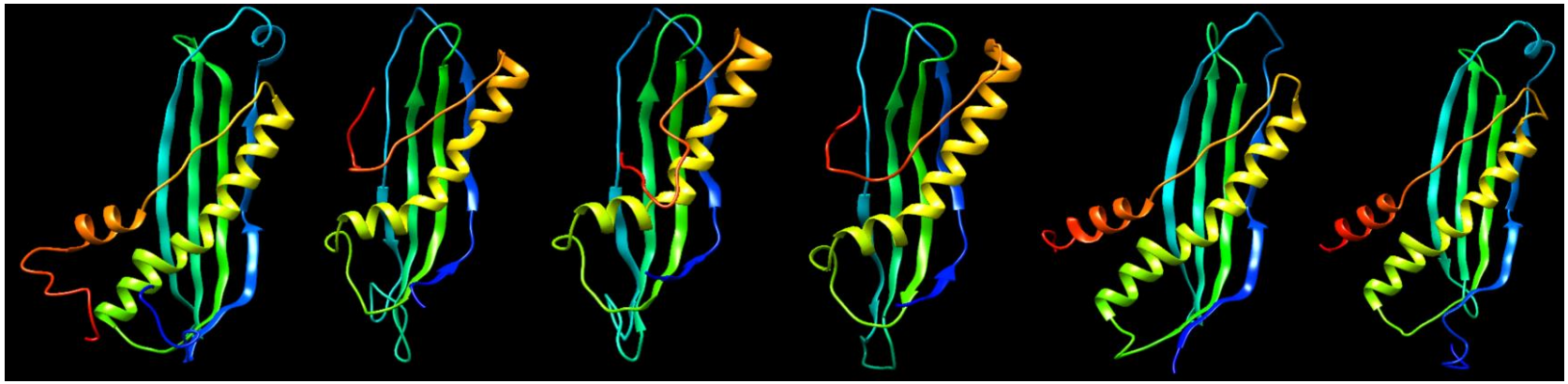
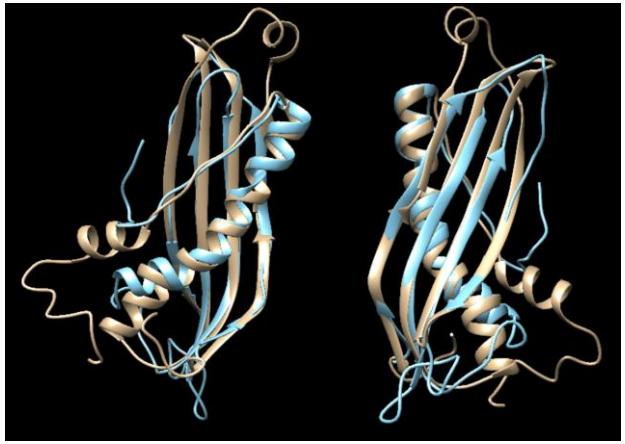
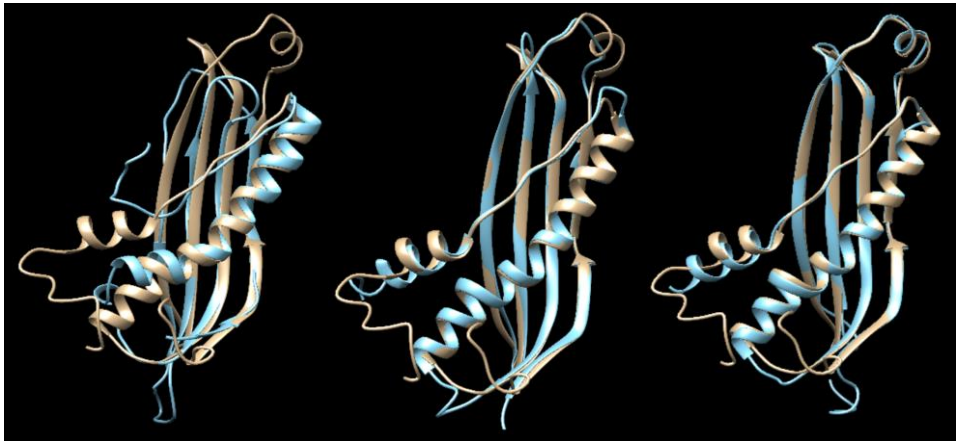


Figure 3. 7. Proposed schematic representation of the components of *L. casei* GCRL163 translocase system showing proteomic changes under prolonged heat stress. Ffh, signal recognition particle (SRP) protein; FtsY, signal recognition particle receptor; LepB, signal peptidase I; YajC, preprotein translocase insertion/stabilisation protein; SecY, preprotein translocase apparatus protein; SecA, Protein translocase subunit SecA; SecB, Uncharacterized BN194_10000; PrsA, foldase protein PRs; DnaJ, chaperone protein DnaJ; GroEL, class I heat-shock protein (chaperonin) large subunit; GrpE, molecular chaperone (heat shock protein, Hsp70 complex); Tig trigger factor Tig. Up-regulated proteins are indicated by red and down-regulated by green arrows with asterisk indicating FDR (**, FDR<1% and *, FDR<5%).



A B C D E F





G H I J K

Figure 3. 8. Modelled 3D-structures of proteins annotated as SecB and homologs with SecB-like superfamily domains (IPR035958) and structural alignment with an *E. coli* SecB. Proteins were modelled using Phyre2 and aligned using UCSF Chimera software. Protein identities (UniProt or NCBI) and lengths (amino acids) are: **A**, *E. coli* K12 SecB P0AG86 (155); **B**, *L. casei* GCRL163, uncharacterised protein (137); **C**, *L. casei* W56, uncharacterised protein K0N3D6 (gene locus BN194_10000) (136); **D**, *L. rhamnosus* GG, conserved protein CAR86774 (gene locus LGG_00879) (135); **E**, *L. plantarum* subsp. *plantarum* ATCC14917 SecB D7V892 (142); **F**, *B. licheniformis* CG-B52, uncharacterised protein A0A2234XY3 (138). Panels **G** to **K** align the *E. coli* SecB P0AG86 template (gold) with the proteins from the following species (blue): **G**, *L. casei* GCRL163, α1-helices central; **H**, *L. casei* GCRL163, view of β-strands; **I**, *L. rhamnosus*; **J**, *L. plantarum* subsp. *plantarum*; **K**, *B. licheniformis*. Ribbon colour: rainbow N to C terminal (red).

Table 3. 3. Fatty acid composition of *L. casei* GCRL163 cells harvested across the growth cycle when cultured at 30 to 45°C

Fatty acids		T^{o} C	30°C			35°C			40°C			45°C		
Systematic names	Common names	FA designation	ML[a]	LL	ST	ML	LL	ST	ML	LL	ST	ML	LL	ST
cis-7-Tetradecenoic acid		14:1 (n-7)c	0.1[b]	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.1	0.0	0.0	0.0
Tetradecanoic acid	Myristic acid	14:0	3.3	3.9	3.1	3.7	2.1	2.5	3.3	2.8	3.2	5.1	3.9	4.8
13-Methyltetradecanoic acid	*iso*-Pentadecanoic acid	i15:0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
12-Methyltetradecanoic acid	*anteiso*-Pentadecanoic acid	a15:0	0.2	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0
Pentadecanoic acid	Pentadecylic acid	15:0	0.2	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.3	0.3
cis-9-Hexadecenoic acid	Palmitoleic acid	16:1 (n-7)c	4.3	4.4	3.5	3.3	3.2	4.0	3.5	3.2	2.9	4.5	3.6	3.0
trans-9-Hexadecenoic acid		16:1 (n-7)t	0.2	2.8	2.2	0.1	2.8	3.2	0.7	2.8	2.7	0.0	0.0	0.0
cis-11-Hexadecenoic acid		16:1 (n-5)	0.5	0.6	0.5	0.3	0.5	0.5	0.3	0.5	0.5	0.0	0.0	0.0
Hexadecanoic acid	Palmitic acid	16:0	15.1	22.3	20.7	10.4	27.0	24.6	10.7	25.5	27.2	26.4	22.1	21.4
		*cyclopentenyl-16:0	0.0	0.4	0.2	0.0	0.4	0.7	0.2	0.4	0.3	0.0	0.0	0.0
14-Methylheptadecanoic acid	*anteiso*-Heptadecanoic acid	a17:0	0.3	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.4
cis-9-Heptadecenoic acid	Margarolic acid	17:1 (n-8)	0.7	0.2	0.2	0.4	0.1	0.1	0.3	0.1	0.2	0.0	0.5	0.3
Heptadecanoic acid	Margarinic acid	17:0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
cis-9-Octadecenoic acid	Oleic acid	18:1 (n-9)	66.4	29.2	33.0	67.7	21.8	17.6	58.7	23.9	20.2	50.2	49.6	55.1
cis-11-Octadecenoic acid	Vaccenic acid	18:1 (n-7)	2.9	28.3	29.0	3.1	32.2	30.0	8.3	25.6	23.1	2.0	4.0	2.3
Octadecanoic acid	Stearic acid	18:0	0.9	1.1	1.2	1.8	1.8	1.8	1.3	2.0	2.0	0.9	1.0	1.1
		*cyclopentenyl-18:0a	0.5	1.4	0.7	1.3	0.6	0.5	1.5	1.0	0.8	0.0	0.4	0.0
		*cyclopentenyl-18:0b	0.4	0.9	0.8	1.3	0.6	0.6	1.3	0.6	0.8	0.0	0.4	0.5
		*cyclopentenyl-18:0c	0.0	0.0	0.1	0.2	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0
		*cyclopentenyl-18:0d	0.6	0.8	0.8	1.2	0.5	0.5	1.3	1.0	0.9	0.0	0.5	0.5
		*cyclopropane-18:0a	2.7	2.1	2.3	4.5	3.2	6.5	7.5	7.3	9.2	10.9	13.5	10.2
		*cyclopropane-18:0b	0.5	1.4	1.3	0.0	2.7	6.5	0.4	2.7	5.4	0.0	0.0	0.0
		Total	100	100	100	100	100	100	100	100	100	100	100	100

[a] Growth phases: ML, mid-log; LL, late log; ST, stationary

[b] Fatty acid composition is expressed as the percentage of each fatty acid relative to the total amount detected in an extract. The Figures represent two independent experiments conducted.

* Full systematic names could not be assigned as full structures were not determined.

3.6 Supplementary Figure

3.6.1 Supplementary Figure 3. 1. Sequence alignment for selected SecB proteins using Clustal Omega (multiple-sequence) and Needle (pair-wise comparisons) (EMBL-EBI, www.ebi.ac.uk) and cladogram constructed in the EMBL-EBI tools suite.

CHAPTER 4

PROTEOMICS OF *LACTOBACILLUS CASEI* GCRL163 SURFACE PROTEINS REVEALS INDUCTION OF PROBIOTIC-LINKED PROTEINS DURING HEAT STRESS WITH IMPACTED BINDING OF HEAT-ADAPTED CELL TO HUMAN COLORECTAL ADENOCARCINOMA HT-29 CELLS

4.1 Abstract

Lactobacillus casei strains are non-starter lactic acid bacteria which improve cheese ripening and may interact with host intestinal cells as probiotics, for which cell surface proteins play a key role. To understand the impact of prolonged heat stress on *L. casei* surface proteins, *Lactobacillus casei* strains are non-starter lactic acid bacteria which improve cheese ripening and may interact with host intestinal cells as probiotics, for which cell surface proteins play a key role. To understand the impact of prolonged heat stress on *L. casei* surface proteins, three complementary methods (trypsin shaving [TS], LiCl-sucrose extraction [LS] and extracellular culture fluid [ECF] precipitation) were employed to obtain proteins and peptides from *L. casei* GCRL163 cells, grown in bioreactors under controlled conditions. Label-free quantitative proteomics was used to characterize the surface heat stress response. A total of 416 proteins, including 300 extra-cytoplasmic and 116 cytoplasmic proteins, were quantified as surface proteins. LS caused significantly higher cell lysis as growth temperature increased. However, most extra-cytoplasmic proteins were exclusively obtained from LS fractions, demonstrating the utility of LiCl extraction of surface proteins in a species producing no S layer. Cell wall hydrolases, adhesins, and homologues of major secreted *L. rhamnosus* GG proteins Msp1/p75 and Msp2/p40, were up-regulated in surface and secreted protein fractions during heat stress, suggesting that cell adhesion in heat-stressed cells could be altered. Hydrophobicity analysis indicated hydrophilicity was enhanced at sub- and supra-

optimal growth temperatures. The binding-capacity of *L. casei* GCRL163 to human colorectal adenocarcinoma HT-29 cells also increased for heat-stressed relative to acid-stressed cells. This study demonstrates that prolonged heat stress influences cell adhesion and abundance of surface proteins, which may impact probiotic functionality.

Keywords: cell surface proteins, trypsin shaving, LiCl, proteomics, prolonged heat stress, cell lysis, cell adhesion, hydrophobicity, *Lactobacillus casei*.

4.2 Introduction

Two major groups of lactic acid bacteria (LAB) are involved in the production and ripening of cheese, a solid-matrix fermented dairy product with high probiotic delivery potential to the gut. These include carefully-selected and added starter LAB, which initiate the fermentation process (Fox *et al*. 2017), and the adventitious non-starter lactic acid bacteria (NSLAB), which contribute to ripening and flavour development (Hickey *et al*. 2017). The NSLAB are made up of several homofermentative or heterofermentative *Lactobacillus* species including *L. paracasei*, *L. plantarum*, *L. casei* and *L. brevis*, as well as pediococci and enterococci (Settanni & Moschetti 2010). The NSLAB have also been demonstrated to interact with and adhere to the host intestinal cells, potentially conferring health benefits as probiotics (Saarela *et al*. 2000). Adhesion to human intestinal cells and their associated extracellular matrix proteins is crucial for intestinal colonization, competitive pathogen exclusion and other mechanisms underpinning the probiotic functionalities of LAB.

Several surface structures of LAB have been implicated in adhesion including lipoteichoic acids (den Camp *et al*. 1985), exopolysaccharides (Fanning *et al*. 2012) and surface proteins (Zhu *et al*. 2016), as well as extracellular proteins (González-Rodríguez *et al*.

2012). Surface proteins may include integral membrane proteins, lipoproteins, cell wall-binding proteins, cell membrane-anchored proteins and anchorless surface or moonlighting proteins (Tjalsma *et al*. 2008). These surface proteins may interact with the host and mediate immunomodulation, chemical sensing and communication or cross-talk (Lebeer *et al*. 2010). 'Moonlighting proteins' may be located in both the cytoplasm and at the cell surface, where they may be involved in mediating probiotic mechanisms, and include glycolytic and related proteins (enolase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, ATP synthase, acetyl-CoA acetyltransferase), DnaK and other chaperonins, ABC transporters (such as OppA), glycosyl hydrolases, flavoproteins and 50S ribosomal protein L10 (Kainulainen & Korhonen 2014). Several *in vitro* model systems have been developed to demonstrate host-bacterial adhesion, including adhesion of lactobacilli to human derived adenocarcinoma cells Caco-2 (Elo *et al*. 1991) and HT-29 (Adlerberth *et al*. 1996; Wang *et al*. 2008), stratified squamous epithelium (Edelman *et al*. 2012) and extracellular matrix (ECM) proteins such as collagens, laminin, mucin and fibronectin (Štyriak *et al*. 2003; Toba *et al*. 1995). The HT-29 cell line is described as a suitable model for *in vitro* study of adhesion in lactobacilli, since it is capable of demonstrating characteristics of mature enterocytes and *in vitro* expression of morphological and functional differentiation (Lesuffleur *et al*. 1990).

The bacterial cell envelope and associated surface proteins are directly exposed to environmental stresses such as temperature extremes, acidity and osmotic fluctuations during product manufacture and subsequent gastrointestinal passage (Papadimitriou *et al*. 2016). Most often, bacteria respond to environmental stresses through modulation of an array of surface proteins to facilitate survival and function (Sengupta *et al*. 2013). However, identification of surface proteins in bacteria with minimal or no contamination from cytosolic

proteins is still of concern. Occurrence of cytosolic proteins in surface protein extracts may be due to cell lysis during handling or autolysis during growth (Espino *et al*. 2014), moonlighting activity (Wang *et al*. 2013) and proteins captured in microvesicles (Domínguez- Rubio *et al*. 2017).

Several strategies have been applied to extracting surface proteins in LAB, which can be grouped into either chemical or enzymatic approaches. Lithium chloride as a chemical method for surface protein extraction has been applied to several *Lactobacillus* species, including *L. casei* (Nezhad *et al*. 2012), *L. salivarius* (Wang *et al*. 2017) and *L. acidophilus* (Klotz *et al*. 2017). However, the LiCl extraction method is still amongst those considered overly harsh for surface protein extraction, as it may lead to leakage of cytosolic proteins resulting in contamination (Johnson *et al*. 2016; Tiong *et al*. 2015). Hussain *et al.* (1999) noted that LiCl extraction might not be the optimum method for selective extraction of surface proteins from *Staphylococcus epidermidis,* as LiCl treatment resulted in DNA release with a reduction in cell viability. In fact, Gram staining of *S. epidermidis* cells treated with LiCl revealed lysis of >50% of cells. Enzymatic methods may involve shaving techniques which utilise enzymes such as trypsin for surface peptide extraction. Trypsin shaving was first used in *Streptococcus pyogenes* (Rodríguez-Ortega *et al*. 2006) and was later applied in Gram-positive bacteria including *B. animalis* subsp. *lactis* (Zhu *et al*. 2016), *S. pneumoniae* (Olaya-Abril *et al*. 2013), *Lactococcus lactis* (Meyrand *et al*. 2013) and *L. monocytogenes* (Zhang *et al*. 2013); in Gram-negative bacteria, including *E. coli* (Walters & Mobley 2009), and in eukaryotic microbes such as *S. cerevisiae* (Braconi *et al*. 2011) and *Schistosoma mansoni* (Castro-Borges *et al*. 2011). Contamination of surface proteins through surface shaving has been attributed to bioinformatic misidentification of cellular localization, cell lysis and the concept of moonlighting proteins (Olaya-Abril *et al*. 2014; Solis & Cordwell

2011; Solis *et al*. 2010; Tjalsma *et al*. 2008). It is therefore expedient to assess membrane integrity following surface protein extraction in order to identify surface-associated proteins (Solis *et al*. 2010). Plate counting methods, although often used, are not a reliable technique for evaluating bacterial cell viability and membrane integrity due to the possible presence of viable but non-culturable cells (Hammes *et al*. 2010). Hence, more robust methods are required for proper assessment of membrane integrity following surface protein extraction.

In this study, we present a label-free quantitative proteomic analysis of cell surface proteins obtained from three protein fractions of whole cells cultured under bioreactor-controlled conditions at 30 to 45°C. The degree of cell lysis was monitored by flow cytometry and DNA fragment analyses. In the proteomic analysis, *L. casei* GCRL163 cells induced the expression of surface proteins involved in cell survival during the prolonged heat stress. Proteins associated with probiotic mechanisms in gastrointestinal and respiratory tract infections were impacted at the cell surface. This investigation represents an approach to using label-free proteomic data obtained from a combination of methods to determine changes in relative abundance of surface proteins, in order to foster an improved understanding of heat stress response at the cell surface of lactobacilli.

4.3 Results

4.3.1 Quantitative analysis of surface protein extracts from *L. casei* GCRL163

Three different approaches were applied to identify and analyse surface proteins of *L. casei* GCRL163 cultured under controlled growth temperatures at pH 6.5, in order to investigate prolonged heat stress responses at the cell surface following prolonged heat stress. Surface-exposed proteins were detected by trypsin shaving (TS) and surface-associated

proteins were extracted by LiCl-sucrose (LS) while proteins released into the growth media or extracellular culture fluid (ECF) were precipitated and concentrated using TCA-acetone. Flow cytometry and DNA fragment analyses were used to investigate the degree of cell lysis following TS and LS treatments. Mean fluorescent intensity (ΔMFI) from the flow cytometry analysis indicated cell lysis of 2.6% and 8.0% in cells treated with TS and LS respectively at 35°C growth temperature. At 45°C, 5.4% and 17.4% cell lysis was estimated respectively, indicating greater cell lysis at elevated temperature and in cells treated with LS, despite the inclusion of sucrose as an osmotic protectant (Figure 4. 1). There was no significant difference in the degree of cell lysis observed in cells grown at 30°C when compared to 35°C (data not shown). DNA fragment analysis also supported the observation that LS treatment led to higher cell lysis compared to TS treatment and that the cells were more susceptible to injury as growth temperature increased (Figure 4. 2). Notwithstanding the observations made, these methods have been used previously to extract surface proteins in *Lactobacillus* species (Espino *et al*. 2014; Johnson *et al*. 2013b).

Proteins obtained by the three methods were subsequently analysed by nano-liquid chromatography and high-resolution tandem mass spectrometry and identified using the Andromeda search engine (refer to Experimental procedures in Chapter 2 for details). A total of 826 and 183 proteins were obtained from the LS and TS fractions respectively and 47 proteins from the concentrated ECF precipitates. The proteomic data were filtered to exclude proteins detected on the basis of a single matching peptide, suspected contaminants and reverse database matches and considering only proteins detected in a minimum of three replicates of any biological sample (Supplementary Table 4. 1). The complete peptide- and protein-level MaxQuant output files are fully reported in Supplementary Table 4. 2. We addressed the challenge of the surface protein contamination by cytoplasmic proteins released

through cell lysis by harvesting the cells at mid-exponential growth phase. Also, different bioinformatic prediction tools, including Gpos-mPLoc (Shen & Chou 2009) and PSORTb server 3.0.2 (Yu *et al*. 2010) algorithms, were used to predict the subcellular locations of the proteins identified in all the protein fractions. Of the 826 and 183 proteins from the LS and TS protein fractions, 290 and 65 respectively were predicted to be extra-cytoplasmic proteins. In addition, 25 proteins of the total 47 proteins identified from the concentrated ECF protein fraction were predicted to be extra-cytoplasmic proteins. Once accounting for overlapping proteins that were detected using all the three methods, a combined list of 300 extra-cytoplasmic proteins with secretory, transmembrane or surface-anchoring motifs was generated (Figure 4. 3A). These data indicate that, despite significant lysis of cells following growth at higher temperatures and treatment with TS or LS, bioinformatic analysis based on their predicted sub-cellular locations revealed a diverse range of surface-located proteins. Furthermore, 99 of the 826 proteins detected in LS were not detected in the corresponding cell lysates obtained by bead beating (cell-free extracts, CFEs) (archived proteomic dataset with identifier PXD007097 in ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al*. 2015) partner repository) and 67% of these displayed secretory or cell surface associated motifs, indicating the utility of LS extraction in detecting proteins that would otherwise be unavailable for evaluating impacts of stress environments.

Of the remaining 536 proteins in LS extracts designated as having a cytoplasmic location, many were highly abundant proteins in the CFEs and were likely present due to lysis. However, selecting proteins for further analysis on the basis of high abundance (LFQ values) may have eliminated proteins of low abundance that were extracted from a surface location by LS. Consequently, we adopted an approach to consider cytoplasmic proteins, which were detected in at least two of the three protein fractions, as cytoplasmic-surface-

associated proteins, resulting in notation of 116 proteins in this category (Figure 4. 3B; Supplementary Table 4. 3). Several of these proteins have been reported extensively in the literature as cytoplasmic proteins performing moonlighting roles at the cell surface, involving in host-bacteria interaction (Huberts & van der Klei 2010; Wang *et al*. 2013). In total, 416 surface proteins were identified in *L. casei* GCRL163, including the extra-cytoplasmic and the designated cytoplasmic-surface-associated proteins (Supplementary Table 4. 4). The identified 416 surface proteins obtained by the three methods were then grouped into cell membrane-anchored (141), integral membrane/transmembrane (72), cell wall-associated/extracellular (87) and cytoplasmic-surface-associated (116) proteins. Among these groups, 24 lipoproteins and 47 proteins possessing N-terminal signal peptides were identified.

4.3.2 Lithium chloride extraction detects more proteins with secretory, cell membrane and cell wall motifs than trypsin shaving

Trypsin shaving, LS and ECF precipitation are considered complementary approaches, as each method is characterized by its enrichment of specific proteins. Here, we found that of the 300 extra-cytoplasmic proteins, the LS, TS and ECF precipitation approaches exclusively detected 227, 3 and 4 proteins, respectively (see Figure 4. 3A). The difference observed in specificity suggests that the three approaches differed in their ability to identify surface proteins associated with the cell envelope. Of the four proteins detected exclusively in the ECF precipitates, three possessed N-terminal secretion signal domains. A comparison of the TS and LS extracts (Figure 4. 4) showed that LS treatment led to the identification of more cell wall-associated, cell membrane and integral membrane proteins and hence more cell surface proteins in *L. casei* GCRL163. Of the 24 lipoproteins and 47 proteins possessing N-terminal signal sequence for secretion, 16 and 22 respectively were exclusively detected by LS, indicating the proteins may have been trafficked to the cell

surface rather than to the external medium. These results demonstrate the effectiveness of LS extraction in identifying the broader surface subproteome of *L. casei* GCRL163 compared with TS.

4.3.3 Functional classification of abundant and differentially modulated surface proteins during prolonged heat stress

The next aim was to characterize relative abundance of cell surface proteins in *L. casei* GCRL163 cells cultured at different growth temperatures under controlled conditions. Some proteins were detected in high abundance at the cell surface in all the protein fractions and these included mostly the glycolytic proteins, ribosomal proteins and molecular chaperones. The most abundant proteins in LS fractions at all growth temperatures were the glycolytic proteins (Gap, Eno, Pyk and Pgk), elongation factor Tuf, DNA-binding protein Hup and chaperone GroEL. In TS fractions, the most abundant proteins included glycolytic proteins (Pgk, Pyk, and Eno), ribosomal proteins (RplL, RpsA, RplB and RplD), molecular chaperones (DnaK and GroEL), elongation factor Tuf and DNA-binding protein Hup. The majority of these proteins have been linked to moonlighting functions, involving in host-bacterial adhesion (Xolalpa *et al*. 2007). Other proteins with potential moonlighting functions that were abundant at the cell surface include protein translocase subunit SecA, which primarily functions in protein secretion, elongation factor Fus, which is associated with protein translation in the cytoplasm, and molecular chaperones GroES and GroEL. The most abundant proteins in the ECF included several functionally uncharacterised peptidases and hydrolases putatively involved in peptidoglycan turnover (BN194_00240, BN194_02560, BN194_02820, BN194_21500, and BN194_23630) as well as putative autolysins (BN194_02430 and YrvJ), the transmembrane protein BN194_29420, and cytoplasmic proteins (GroEL, Tuf).

To identify temperature-related changes in the cell surface sub-proteome, we considered the abundance of proteins (LFQ) identified at each growth temperature relative to their expression at 30°C ($\geq \log_2$ 1.0-fold, up-regulated or $\leq \log_2$ -1.0-fold, down-regulated at p <0.05, FDR <5%). Functional group analysis was performed based on protein COG (Tatusov *et al*. 2000) and KEGG (Kanehisa & Goto 2000) classification. From the total of 416 surface proteins, 57, 59 and 194 surface proteins were differentially expressed at 35°C, 40°C and 45°C, respectively, in the LS protein fraction (Figure 4. 5A and Supplementary Table 4). Also, 52, 82 and 127 in the TS and 17, 24 and 16 proteins in the ECF fractions were differentially expressed at 35°C, 40°C and 45°C respectively (Figure 4. 5B and C). The most differentially up-regulated proteins in the LS protein fraction at 35°C and 40°C were proteins involved in lipid-related metabolism including FabK (BN194_22580), BN194_01080, AcpP_2 (BN194_22590), BN194_22500 and ABC transporter (superfamily ATP binding cassette) BN194_25870. At 45°C growth temperature, uncharacterized proteins BN194_15370 and BN194_11770, phosphotransferase systems (PTS) protein BN194_28560 with PTS EIIB domain (L-Asc family), and glycosyltransferase BN194_13290, were the most up-regulated. Furthermore, a group of proteins could not be detected in the 30°C control but were abundantly expressed at higher temperatures in the LS fraction and included uncharacterized protein isoform BN194_27750/BN194_27760, a putative α-L-fucosidase, PTS proteins BN194_07420 and LevE_4, putative ABC hemin import ATP-binding protein HrtA_2 (BN194_25880) and uncharacterised protein BN194_20240. In the TS protein fraction, the most up-regulated proteins at 35°C and 40°C included the CHAP/SibA-domain containing cell wall hydrolase BN194_00240, septum site-determining protein DivIVA, heat-shock protein serine protease HtrA/DegP, ribosomal protein RplX and NlpC/P60 domain surface antigen protein BN194_21500. At 45°C, XpaK (BN194_01710) involved in the

pentose phosphate pathway, PspC domain-containing protein YthC (BN194_10300), ribosomal proteins RplW and RpsQ, and ATP-dependent Clp protease ClpP_2 became significantly more abundant. The pattern of regulation in the cell surface fraction was slightly different from our previous observation in the CFEs where protein ThiM (BN194_03200), related to cofactor metabolism, was the most up-regulated at all the growth temperatures. Furthermore, proteins associated with protein folding/turnover (BN194_29440) and nucleic acid/nucleotide metabolism (NrdD, BN194_01250), were other most abundantly expressed at 35°C in the CFEs (Chapter 3). ABC-type transporter (BN194_21220), proteins in the PTS (FruA3 and BglP), carbohydrate-related metabolism (AdhE), amino acid-related metabolism (MetE) and Chaperones (GroEL and DnaJ) constituted other most abundantly expressed at 40°C and 45°C in the CFEs.

Several differentially expressed proteins were related to different metabolic and molecular processes, which could impact on the physiological state of the cells. These include cell wall biogenesis, cytokinesis, lipid metabolism, co-factor syntheses, protein folding and recycling, cell defense, phosphotransferase and ABC- and PTS-type transporter systems, central glycolytic and intermediary pathways, carbohydrate metabolism, ribosomal proteins, tRNA/ribosome assembly, post-translational modification, tRNA aminoacyl synthesis, and transcriptional regulation. These findings demonstrated that *L. casei* GCRL163 cells elicited a complex network of events during prolonged heat stress at the cell surface, probably related to protecting cells from functional and structural damage during heat stress.

4.3.4 Cell wall biogenesis, cell wall-anchoring and related proteins are modulated during prolonged heat stress

Several proteins, associated with cell wall biogenesis or turnover, were detected in the LS, TS and ECF protein fractions and were impacted by prolonged heat stress (Table 4. 1). Most of the cell wall hydrolases detected were consistently enhanced at 35°C and 40°C while they were repressed at the elevated temperature of 45°C relative to 30°C. The cell wall hydrolases over-expressed in the LS and ECF protein fractions included a protein (BN194_02820) with NlpC/P60 and SLAP (S-layer binding) domains and a 43 kDa putative cell wall protein BN194_00240, which possesses a CHAP/SibA domain. In the TS and ECF fractions, NlpC/P60 protein BN194_21500 was up-regulated at 35°C and 40°C. However, some cell wall hydrolases were repressed at 45°C in the TS (BN194_02820 and BN194_23630) and ECF (BN194_00240 and BN194_02560) protein fractions. The enhanced expression of the cell wall hydrolases in the mid-exponential cells, grown at 35°C and 40°C, suggests increased peptidoglycan turnover and cell division during rapid cell growth while growth is impaired at the elevated growth temperature of 45°C. This is consistent with the observed impeded growth and early movement into stationary phase during culture at 45°C (Chapter 3).

Furthermore, other surface proteins involved in peptidoglycan synthesis, decoration and turnover were detected mostly in the LS and TS protein fractions and were impacted by prolonged heat stress. MurE (BN194_02160) and GlmU (BN194_26940), involved in early peptidoglycan biosynthesis, were over-expressed at 45°C and all growth temperatures relative to 30°C in the TS fractions. However, expression of these proteins was repressed in the CFEs at 45°C (Supplementary Table 4. 4). Some proteins associated with peptidoglycan polymerization in late peptidoglycan synthesis were inhibited at 45°C either in the CFEs, TS or LS protein fractions, including DacA, PbpF, PbpA/YqgF and PonA. Other proteins associated with cell wall hydrolysis including glycosylhydrolases, such as peptidase

BN194_21320 and cell wall hydrolase BN194_02430, were also repressed at 45°C. The putative autolysin BN194_02430, likely involved in peptidoglycan catabolism through lysozyme activity, was over-expressed at 35°C and 40°C relative to 30°C in all the protein fractions while YrvJ (BN194_17200), involved in septum splitting and potentially autolysis, was up-regulated at 35°C and 40°C in LS and ECF. Proteins involved in cytokinesis, including cell shape-determining proteins such as MreC and MreB, were repressed in the LS and CFEs but up-regulated in the TS at 45°C while Mbl, a MreB-like protein, was repressed at 35°C and 40°C in the TS protein fractions. Proteins associated with septation formation, including DivIVA (BN194_14800, increased in all growth temperatures in the TS but repressed at 45°C in the LS and no abundant change in the CFEs) and PbpB (BN194_14700, increased at 35°C and 40°C in the LS), were also impacted by heat stress. Others including septation ring formation regulators EzrA (BN194_14460), FtsX (BN194_10200, increased in the CFEs at 40°C and 45°C) and FtsZ (BN194_14760) were inhibited at 45°C in the LS with FtsZ repressed at all the growth temperatures in the TS protein fraction (Supplementary Table 4. 4). However, FtsE was over-expressed at 45°C in the TS fraction. These findings suggested that cell septation was impacted by heat stress and further support the contention that peptidoglycan turnover is enhanced during rapid growth at 35-40°C but impaired at 45°C.

Other proteins involved in the synthesis of surface structures, including rhamnose-containing exopolysaccharides, were over-expressed in the TS at all growth temperatures (RmlB, RmlB_2), 35°C (SpsK, SpsK_2), 40°C and 45°C (RmlA, RmlA_2) relative to 30°C (Supplementary Table 4. 4), suggesting increased exopolysaccharide synthesis. However, Mrp chromosome partitioning ATPase BN194_21890, putatively involved in exopolysaccharide synthesis, was less abundant at 45°C in the LS and CFE protein fractions.

Integral membrane protein LtaS1 (BN194_09110), involved in lipoteichoic acid synthesis, was repressed at 45°C in the LS and GalE, involved in teichoic acid synthesis for peptidoglycan decoration, was repressed at 35°C and 45°C in the TS fractions. Protein subunits of the complex for D-alanylation of teichoic acids including DltC, involved in linking the surface structures onto the cell surface, was up-regulated at 35°C while DltD was inhibited at 45°C in the LS fractions. Cell wall LPXTG motif anchor domain-containing protein BN194_05290 was up-regulated in the LS at 35°C but repressed in the TS and ECF protein fractions at 45°C. WxL domain cell surface protein BN194_01540 was up-regulated at 35°C and 45°C in the ECF protein fractions. Sortase BN194_22780 was up-regulated at 45°C in LS but repressed in TS and ECF protein fractions.

4.3.5 Impact of prolonged heat stress on surface proteins involved in substrate uptake and translocation

Several proteins in ATP-binding cassette (ABC) transporter systems, predicted to be extra-cytoplasmic proteins, were detected in the protein fractions (Figure 4. 5 and Supplementary Table 4. 4). The peptide transporting proteins, detected variously in TS and LS fractions, showed mostly inhibited expression and included OppA (BN194_17880), OppA_2 (BN194_20640), OppB (BN194_17900), OppC_2 (BN194_20620), OppD_2 (BN194_20600) and DppE_4 (BN194_22020). OppA_2, OppB, OppD_2, DppE_4 and DppE_3/OppA (BN194_14020) were also repressed in the CFEs at 45°C. Similarly, MetQ_2 (BN194_13740) and MetN (BN194_13750), involved in methionine uptake, were detected in the LS protein fraction and only MetQ_2 was repressed at 45°C (up-regulated in the CFEs at 40°C and 45°C) while OpuAA (BN194_22850), associated with glycine betaine uptake, was repressed at all the growth temperatures. However, cell membrane-anchored ABC-type transporters GlnQ_2, GlnQ_3, GlnQ_4, involved in glutamine uptake, and PotA_2 and PotD,

associated with polyamine uptake, were mostly up-regulated in the surface protein and CFE fractions, suggesting an increased uptake of isoglutamine and polyamine, possibly released from peptidoglycan turnover during growth as well as from medium sources.

Other surface proteins impacted by the heat stress and detected only in the LS protein fraction include proteins involved in Mn/Zn ion uptake (FimA and MntB, both were repressed), phosphonate uptake (PhnC_2, increased at 35°C and 40°C), phosphate uptake (PstBA and PstBB, both repressed at 45°C) and metal ion uptake (BN194_25510, increased $\log_2$ 1.1-fold at 45°C) (Supplementary Table 4. 4). Proteins BN194_13430 and BN194_13440, annotated in the sequenced genome of *L. casei* GCRL163 as Lipid A export related, were detected in the LS protein fraction as up-regulated at 45°C ($\log_2$ 1.8-fold) but only protein BN194_13440 was detected in the TS protein fraction and also enhanced at 45°C ($\log_2$ 1.4-fold). Furthermore, surface associated ABC-type transporters involved in the transport of unknown substrates showed overall repressed expression in the TS and LS fractions with the exception of BN194_07700 and BN194_07710 which were enhanced in the TS at 35°C and ECF at 40°C.

The core PTS protein PtsH was up-regulated at all growth temperatures in the TS and at 45°C in the LS and CFEs while PtsI was repressed at 45°C in the LS fractions. The third core component of the PTS system, HprK, was previously detected in CFES, where it was up-regulated at 45°C (Chapter 3). Several predicted extra-cytoplasmic proteins, involved in PTS, were detected mostly in the LS protein fraction and several were co-located in the genome, forming all components of a sugar transport system. Although expression levels of the surface proteins when detected in the LS fraction generally reflected the regulatory patterns when seen in the CFE, in the case of the sugar-PTS systems this was not always the

case, so proteins were examined in all of the fractions prepared at the different temperatures. Cell membrane-anchored protein FruA_3, a phosphoenolpyruvate (PEP)-fructose/mannose family system, showed repressed expression at 35°C and 45°C in the LS while seen as highly more abundant at 45°C in the TS and CFE fractions. This protein is co-located in the genome with FruK (1-fructokinase/tagatose-6-phosphate kinase), which was similarly up-regulated in the CFE at 45°C (Chapter 3). In the phosphoenolpyruvate (PEP)-glucose phosphotransferase system (PEP-PTS), integral membrane protein BglP (BN_194_06940), involved in β-glucoside uptake, was repressed at 35°C and 40°C in the LS fraction although seen as up-regulated in the CFE at 40 and 45°C (Supplementary Table 4. 4). Three surface proteins involved in mannose uptake ManX_5 (BN194_29720), SorA_4 (BN194_29710) and ManZ_9 (BN194_29700), were similarly regulated in the LS fraction, all showing lower abundance at 45°C. However, in the CFE, these three genomically-linked proteins were up-regulated at 40°C and 45°C. LevE_4, the IIA/B component of a fructose/mannose PTS system, was enhanced at 45°C in TS whereas the IIC and IID components of this system were not detected in any fraction, including the CFE. All four proteins making up another fructose/mannose/sorbose PTS system, LevD/ManX (BN194_03000), LevE/ManX (BN194_02970), LevG/ManZ (BN194_02990) and LevF/ManY (BN194_02980), were detected in the LS fraction with only LevD/ManX increased $\log_2$ 1.0-fold and 1.7 at 35°C and 45°C; three of the four proteins were more abundant at 40°C in the CFE. Cell membrane-anchored protein PTS ascorbate family subunit IIB (BN194_28560) was increased by $\log_2$ 5.8-fold at 45°C in the LS protein fraction and was not detected in the CFE. These observations, firstly, emphasize the necessity of reviewing data across different protein fractions, surface and cytosolic, to inform conclusions about altered abundance in cells from different growth conditions and, secondly, demonstrate greater transport capacity for sugars at elevated growth temperatures.

4.3.6 Induction of surface proteins involved in signal transduction, transcription, translation and protein synthesis in *L. casei* GCRL163 under prolonged heat stress

Proteins associated with tRNA and ribosome processing, such as RbgA (BN194_15940) and RnpA (BN194_30620), were up-regulated while TrmK and CshA were repressed at 45°C (Figure 4. 5). Predicted extra-cytoplasmic proteins, such as TrxA_2, TypP and tyrosine/serine phosphatase BN194_29310 involved in post-translational modification, showed relative abundance in the LS at 45°C. The expression of these proteins was different in the CFEs where they were down-regulated at 45°C (Supplementary Table 4. 4). Proteins involved in tRNA aminoacyl synthesis, including LysS, ThrS, GlyS, GatA and Zinc ribbon domain containing protein BN194_11890 were repressed mostly at 45°C in the LS and CFEs. Proteins lytR_3, LytR_4 and TetR family transcriptional regulator BN194_12190 were repressed at 45°C in the LS fraction. Similarly, PurR2, XRE family transcriptional regulator BN194_23510 and YebC/PmpR family BN194_11600 demonstrated repressed expression at 45°C in the TS fraction. We observed that large subunits (LSU) of 50S ribosomal proteins (L32, L33 and L35), which were predicted to be extra-cytoplasmic proteins as well as cytoplasmic proteins, showed enhanced expression at 45°C in the LS protein fraction. Other ribosomal proteins that were enhanced at 45°C include L2, L4, L17, L20, S11 and S18 which were detected in the TS protein fraction.

4.3.7 Induction of surface proteins associated with protein quality control machinery under prolonged heat stress

Prolonged heat stress led to protein folding molecular chaperones and proteases involved in protein turnover to be differentially expressed in the protein fractions (Figure 4. 5 and Supplementary Table 4. 4). ATP-dependent Clp protease ATP-binding subunits such as

ClpB, ClpB_2, ClpC and ClpE and molecular chaperones GroEL and GroES were over-expressed mostly at 40°C and 45°C in the surface fractions, similar to the expression pattern in the CFEs. DnaJ was over-expressed at all growth temperatures in TS but only at 45°C in LS while DnaK was not differentially modulated in the fractions except in the CFEs where they were differentially induced at 45°C. GroES, GroES, DnaK, Tig and HtrA/DegP were abundant in the ECF fraction. HtrA/DegP with enhanced expression at 40°C and 45°C in the CFEs, was up-regulated at all growth temperature in TS and ECF, and only at 45°C in LS while trigger factor Tig was enhanced at 40°C and 45°C in the TS. However, foldase protein PrsA, predicted to be a lipoprotein with a N-terminal signal peptide for secretion, was repressed in TS (log_2 3.9-fold) and LS (log_2 1.0-fold) but up-regulated in the CFEs at 45°C. Other proteins exhibiting lower abundance include α-crystallin domain heat shock protein Hsp18, which was repressed at all growth temperatures in the LS and GrpE at 45°C in the TS protein fractions. The other α-crystallin domain heat shock protein Hsp20 (BN194_29440) was repressed at 40°C (log_2 1.4-fold) but enhanced at 45°C (log_2 1.7-fold) in the LS fraction. BN194_29440 was one of the most abundantly expressed proteins at all growth temperatures in the CFEs.

4.3.8 Other cell surface proteins modulated by prolonged heat stress

Results from our proteomic data further revealed differential expression of several other surface proteins (Figure 4. 5 and Supplementary Table 4. 4). The cell membrane-anchored ATP synthase subunits such as AtpA and AtpF were up-regulated at 35 to 45°C while AtpD was only enhanced at 45°C relative to 30°C in the TS fraction. The surface protein YajC, associated with bacterial translocase systems was repressed at 40°C and 45°C while SecA and Ffh were up-regulated at 45°C in the TS. We also detected differential expression of proteins associated with fatty acid and phospholipid synthesis at the cell surface

and some of these were up-regulated at 35°C and 40°C (BN194_22500, BN194_22510, FabK, FabF and AcpP2) mostly in the LS. Surface proteins associated with cell defense and detoxification, including Npr, DkgB and Gpo, were up-regulated in the LS with YfeX showing repressed expression in the LS and TS fractions at 45°C. Some glycolytic enzymes (Tpi, and Pgi) were up-regulated while others (Fba_2 and Gap) were repressed at 45°C in the surface fractions. PspC domain-containing protein YthC, putatively associated with bacterial adhesion, was relatively more abundant at all the growth temperatures above 30°C in the TS protein fraction. Cell membrane-anchored protein YheA with YmcA-like domain and cell wall-anchored protein Veg (BN194_27010), involved in biofilm formation, were repressed at 45°C in the surface protein fractions, suggesting heat stress may reduce surface associated growth. Several other extra-cytoplasmic proteins annotated as 'general prediction' and 'hypothetical/uncharacterized' with unknown functions were differentially expressed (Supplementary Table 4.4). Several of these proteins were abundantly expressed at growth temperature of 45°C in the LS and TS fractions. Some of these proteins possessed gene ontology (GO) annotations while others could only be assigned putative identification using BLASTN (Consortium 2016).

4.3.9 *Lactobacillus casei* GCRL163 demonstrates low hydrophobicity at optimum growth temperature that increases for cells cultured at suboptimal or supraoptimal temperatures

The relative abundance of several proteins detected in this study, mostly at 40°C, including cell wall hydrolases (50 kDa SLAP domain-containing NlpC/P60 protein BN194_02820 and a 43 kDa CHAP/SibA domain-containing protein BN194_00240), proteins associated with cell surface structures such as exopolysaccharides (isoforms SpsK, SpsK_2 and RmlA, RmlA_2), cell adhesion (PspC-domain protein YthC), and cytoplasmic

proteins associated with moonlighting functions (Gap, Eno, Pyk and Pgk, Tuf, Hup GroEL and SecA) (Supplementary Table 4. 4), suggested that prolonged heat stress might enhance cell adhesion.

To gain further insight into the adhesive properties of *L. casei* GCRL163 and how temperature of culture may impact on cell adhesion, we used the MATH test to investigate the hydrophobic/hydrophilic and Lewis acid-base properties of *L. casei* GCRL163 cell surface following growth at different temperatures. As shown in Table 4. 2, *L. casei* GCRL163, suspended in low ionic-strength Tris-HCl buffer (40 mM, pH 7.0), was observed to be moderately hydrophilic at all growth temperatures. Cell surface hydrophobicity could be determined from the percentage of bound cells to hexadecane with cells bound >55% considered as strongly hydrophobic, 30-54% moderately hydrophobic, 10-29% moderately hydrophilic and <10% strongly hydrophilic (Chae *et al*. 2006). In *L. casei* GCRL163 cells, cultured to mid-exponential growth phase in bioreactors with set pH of 6.5 at 30°C, 27.1% adhesion to hexadecane was observed. At 35°C, a reduction to 16.3% was observed. The binding to hexadecane increased to 28.6% at 40°C and became slightly reduced to 24.7% at elevated growth temperature of 45°C. These findings revealed that the binding to hexadecane was higher in cells cultured at 30°C, 40°C and 45°C than growth temperature of 35°C, which was close to the optimal temperature of 37°C for the strain (Chapter 3). This suggests that, at optimal growth temperature in the culture conditions used, *L. casei* GCRL163 cells are significantly (p <0.05) less hydrophobic and more hydrophilic.

Also, *L. casei* GCRL163 was evaluated for affinity to chloroform (Lewis acid and electron acceptor) and diethyl ether (Lewis base and electron donor). The stronger affinity to chloroform than diethyl ether indicated strong electron donor and weak electron acceptor

capacity, consistent with the observed hydrophilic cell surface property of this strain. The presence of chemical groups such as $-COO^-$ and $-HSO_3^-$ on bacterial cell surfaces is attributed to a strong electron donor property (Pelletier *et al*. 1997). The Lewis acid-base characteristics is based on comparing the affinity of the cells for a monopolar solvent (Lewis acid or Lewis base) with an apolar solvent that has similar van der Waals forces (Bellon-Fontaine *et al*. 1996). Adhesion to both chloroform and diethyl ether was higher at growth temperatures that were lower (30°C) and higher (40°C and 45°C) than 35°C. These findings demonstrate the non-acidic nature of *L. casei* GCRL163 cell surface with the cells becoming more hydrophilic at growth temperature close to the optimum compared to lower or higher growth temperatures, suggesting that the cell binding capacity to host cells may be impacted by growth at different temperatures.

4.3.10 Adhesion to HT-29 cells is enhanced in heat-adapted *L. casei* GCRL163 compared to acid-adapted cells

We further investigated the adhesion of *L. casei* GCRL163 and three other *Lactobacillus* strains grown at 40°C and control 30°C to HT-29 cells. *L. casei* GCRL163 response to prolonged heat stress at 40°C included the induction of stress responses vital for cellular survival while down-regulating several proteins, inducing some stress responses similar to stationary-growth-phase stress responses and rerouting metabolic pathways at 45°C (Chapter 3). As a result of this observation, coupled with an increased expression of cell wall hydrolases and some moonlighting proteins earlier observed, adhesion was performed on the *Lactobacillus* strains cultured at 40°C.

Adhesion of *Lactobacillus* strains to HT-29 cells varied considerably between the strains and strain specificity was observed (Figure 4. 6). *L. helveticus* K1, originally isolated

from all-natural yoghurt, demonstrated highest adhesion, followed by *L. rhamnosus* NBRC3425 isolated from no-fat yoghurt. *L. casei* GCRL163 from Cheddar cheese demonstrated moderate adhesion while poor adhesion was observed with *L. paracasei* 7K07A2 isolated from natural yoghurt (Figure 4. 7A). Prolonged heat stress of *L. casei* GCRL163 at 40°C led to increased adhesion compared to 30°C in cells cultured at pH of 6.5 and 4.5 (Figure 4. 7B). Moreover, *L. casei* GCRL163, cultured at pH 6.5 to mid-exponential growth phase, adhered more to the HT-29 cells than cells cultured at pH 4.5. The adhesion was also observed to be cell concentration-dependent with highest binding observed at 10^{12} CFU/mL in all the tested strains. These results demonstrated that prolonged heat stress of *L. casei* GCRL163 led to improved adhesion to HT-29 cells while adhesion was reduced in acid-adapted *L. casei* GCRL163 harvested at mid-exponential growth phase.

4.4 Discussion

The current study investigated changes in the abundance of surface proteins and the impact on key metabolic processes in the Cheddar cheese isolate *L. casei* GCRL163 when under prolonged heat stress. Previous extraction of surface proteins from *L. casei* treated with LiCl was reported to generate few proteins, most of which were identified as cytoplasmic proteins (Johnson *et al*. 2016). Using three different approaches however, we detected several proteins in the surface protein fractions obtained from the *L. casei* GCRL163, which were predicted extra-cytoplasmic proteins by the subcellular localization prediction algorithms (Emanuelsson *et al*. 2000). Surface protein fractions from the present study were subjected to processes of concentration and precipitation. This present study further established the suitability of the TS and LS treatments in the enrichment of cell surface protein fractions in non-S-layer producing *L. casei* GCRL163 and effectiveness of the concentrated ECF precipitation by TCA-acetone for obtaining secreted and mechanically shed surface proteins.

Also, we have demonstrated that the *L. casei* cell surface subproteome is probably more diverse than previously reported (Bauerl *et al*. 2010; Munoz-Provencio, Perez-Martinez & Monedero 2011; Nezhad *et al*. 2012) with a complex network of events triggered during prolonged heat stress. Our investigation highlighted specificity in preferential detection of lipoproteins and integral membrane proteins by LS compared to TS extraction. This may be due to the TS only accessing the surface-exposed protein regions whereas LS is able to release lipid-anchored proteins and other proteins associated with the cell envelope. We also suspected that in our prior studies (Nezhad *et al*. 2012), some proteins with adhesive properties were lost during concentration using osmotic removal of solvent. Ethanol precipitation, used in this study, was a faster and more efficient procedure to recover cell surface extracts following LS treatment, as an improvement to previously described protocol on LiCl treatments (Frece *et al*. 2005; Nezhad *et al*. 2010).

Although cell lysis occurred in *L. casei* GCRL163 during the TS and LS treatments, which increased with higher growth temperatures, surface protein extracts with reduced cytoplasmic protein contamination were obtained by the TS approach. Cell lysis has been associated with surface protein extraction using trypsin shaving (Solis & Cordwell 2011) or LiCl (Johnson *et al*. 2016; Tiong *et al*. 2015) treatment in bacteria. Moreover, several reports have demonstrated that minimal cytoplasmic protein contamination during trypsin shaving of surface-exposed proteins is unavoidable as an estimated ≤2% cell lysis is enough to contaminate surface protein extracts during extraction (Köller *et al*. 2008). Cytoplasmic proteins could be present functionally on the cell surface as a result of moonlighting phenomenon by yet unknown export mechanisms (Olaya-Abril *et al*. 2012). Recently, a finding on the production of microvesicles carrying cytoplasmic and cell surface-associated

proteins in *L. casei* BL23 was reported (Domínguez-Rubio *et al*. 2017). This could re-define the models for export mechanisms of moonlighting proteins and other proteins with unidentified secretion systems in bacteria, as several protein orthologs of the microvesicle content were identified in our current proteomic data, including cytoplasmic proteins LacD2, Ldh, PepC2, GroES, GroEL, GpmA2, Eno, Pgk and Gap.

Bacterial surface (S)-layers are produced by several lactobacilli and have been described as crystalline arrays of self-assembling, lattice-like, proteinaceous subunits constituted by S-layer proteins which completely cover the cell surface (Hynönen & Palva 2013; Johnson *et al*. 2013b). Johnson *et al*. (2016) reported that the S-layers, which formed an important structure that anchored secreted proteins to the cell surface in *L. acidophilus* homology group, were not produced by *L. casei*. In the current study, the SLAP domain in NlpC/P60 protein BN194_02820 likely possesses similar attributes as the silent S-layer protein gene *slpB* in *L. acidophilus* ATCC4356 (Boot *et al*.1995). This SLAP domain may assist the 50 kDa NlpC/P60 protein BN194_02820 to bind to S-layer of other *Lactobacillus* species. The 50kDa cell wall hydrolase BN194_02820 NlpC/P60 and 43 kDa hypothetical cell wall protein BN194_00240 CHAP/SibA domain detected in the current study possess sequence similarities to a reported 75 kDa cell wall hydrolase NlpC/P60 (p75) and 40 kDa cell wall hydrolase CHAP domain (p40) respectively, identified in the culture supernatant fluid of *L. rhamnosus* GG (Yan *et al*. 2002) and also detected in the cell surface and culture medium of other closely related *L. casei* BL23 and *L. paracasei* (Bäuerl *et al*. 2010). The protein p75, renamed as major secreted protein Msp1 in *L rhamnosus* GG, was reported to possess a predicted molecular weight of 48 kDa but identified on SDS-PAGE at 75 kDa as a result of glycosylation, which accounted for the difference in weight (Latousakis & Juge 2018; Lebeer *et al*. 2012). The presence of these cell wall hydrolases in the LS, TS and ECF

protein fractions in the current study has further revealed that they are not only secreted but also surface proteins in *L. casei* GCRL163. Furthermore, p75 and p40 have been associated with anti-apoptotic and cell protective activities on intestinal epithelial cells and reduced adverse impact of hydrogen peroxide on epithelial barrier function (Seth *et al*. 2008; Yan *et al*. 2007; Yan & Polk 2002, 2012). Moreover, cell wall hydrolase with CHAP or NlpC/P60 domain plays vital role in cell separation as bacterial autolysins in *Lactobacillus* spp., (Layec *et al*. 2008). Upregulation of these cell wall hydrolases at 35°C and 40°C suggested improved probiotic activities and daughter cell separation. However, extremely elevated growth temperature of 45°C inhibited synthesis, which is consistent with the observed slower growth at this temperature and rapid onset of stationary phase.

The increased expression of autolysins at 35°C and 40°C, including protein BN194_02430 with a peptidoglycan autolysin glycoside-hydrolase-lysozyme domain and cell wall hydrolase/autolysin with cat/SH3-like domain BN194_17200, further suggests that cell wall maintenance, cell division and peptidoglycan autolysis are vital in the adaptive mechanism to cope with increased growth at these temperatures. The reduced abundance of BN194_02430 and BN194_17200 at 45°C is consistent with the reduced growth rate observed at this elevated temperature (Chapter 3). Regulated activities of peptidoglycan/cell wall hydrolases, especially autolysins, are important due to their involvement in cell division and growth, probiotic activities, peptidoglycan remodelling and turnover, invasion and autolysis (Regulski *et al*. 2012). In an uncontrolled condition, peptidoglycan hydrolases would become autolysins, causing bacterial lysis (Chao *et al*. 2013). Despite culturing in a rich medium (MRS), originally containing 2% glucose and then supplemented with 1% glucose, the glucose levels were not depleted as expected, suggesting an alternative carbon source. Intriguingly, the enhanced synthesis of cell wall hydrolases may suggest increased

peptidoglycan and exopolysaccharide degradation with the release of amino acid and nucleotide sugar derivatives, which can be utilized by the bacteria as carbon and nitrogen sources. This is consistent with the increased expression of ABC- and PTS-system surface proteins associated with the uptake of isoglutamine and sugars. Although the PTS-systems are annotated as specific for transport of particular sugars, the majority of the fructose/mannose/sorbose transporters have broad substrate specificity and are known to transport glucose, mannose, fructose, glucosamine and N-acetylglucosamine in several bacteria (Saier 2015). The observed heightened expression of some surface hydrolases at 45°C at the surface, together with greater abundance of sugar-PTS systems detected at the surface and in CFEs, suggests carbon and nitrogen scavenging from cell surface structures occurs during prolonged heat stress.

Abundant expression of molecular chaperones during prolonged heat stress in different protein fractions in this study is consistent with an increased need to protect the cells from adverse effects of heat stress that typically manifests by inducing protein misfolding, denaturation and aggregation in rapidly growing cells (Bukau *et al*. 2006). Some molecular chaperones such as DnaK, GroES, GroEL and Tig have been associated with additional roles at the cell surface. For instance, DnaK demonstrated a low immunogenicity property (Ling *et al*. 2004) and was demonstrated to bind plasminogen (Xolalpa *et al*. 2007). Mucin-binding activity of GroEL in *L. johnsonii* La1 (Bergonzelli *et al*. 2006) and pathogen exclusion potential of Tig in *L. fermentum* RC-14 have been documented (Heinemann *et al*. 2000). The membrane foldase protein PrsA is an extracellular lipoprotein characterized as extracytoplasmic folding factor in *Bacillus subtilis* (Wahlström *et al*. 2003), possessing N-terminal signal peptide and hence can be secreted through the Sec pathway. Two cell wall-associated α-crystallin domain proteins Hsp20 (BN194_29440) and Hsp18 (BN194_07570)

were identified. Intriguingly, Hsp20 demonstrated enhanced expression while Hsp18 was repressed by heat stress, suggesting Hsp18 was not mobilized during prolonged heat stress, possibly to conserve energy. The α-crystallin domain proteins are mobilized for the prevention of irreversible protein aggregation during heat stress (Narberhaus 2002).

Noteworthy, several cytoplasmic proteins were identified as surface associated proteins in the study. Among this group of proteins is a DNA-binding protein HU (Hup). Protein Hup is involved in DNA topology change and stabilization by wrapping DNA, preventing it from denaturation by extreme environmental stress (Anuchin *et al*. 2011). Deletion of the Hup homolog located extracellularly in *Mycobacterium smegmatis* showed increase sensitivity to stress conditions, suggesting the involvement of this histone-like protein in stress response (Whiteford *et al*. 2011). The cytoplasmic proteins, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, tagatose-6-phosphate kinases and tagatose 1,6-diphosphate aldolase 2, were expressed on the cell surface in *L. casei* GCRL163 during prolonged heat stress. The majority of these proteins have been associated with moonlighting functions, contributing to host-bacteria interaction (Wang *et al*. 2013). The abundance of moonlighting proteins and upregulation of cell wall hydrolases linked to probiotic activities, such as the 50 kDa cell wall hydrolase BN194_02820 NlpC/P60 and 43 kDa hypothetical cell wall protein BN194_00240 CHAP/SibA domain, and adhesins such as cell membrane-anchored PspC-domain protein YthC at the cell surface in *L. casei* GCRL163 during heat adapation suggested improved cell adhesion and probiotic functionality. PspC mediates adhesion of *Streptococcus pneumoniae* to host cells and modulates host immunity, thereby playing a vital role in respiratory infeaction (Hammerschmidt *et al*. 2007; Moreno *et al*. 2012; Villena *et al*. 2011). Furthermore, an increase in the abundance of YthC may indicate the importance of *L. casei* GCRL163 in modulating host immunity against gastrointestinal and

respiratory tract pathogens under prolonged heat stress. Upregulation of Rml family proteins involved in the synthesis of exopolysacharide precursors could further alter cell adhesion.

The moderate adhesion to HT-29 cells that was demonstrated by *L. casei* GCRL163 could be linked to its hydrophilic cell surface as determined by the MATH test. The adhesion of LAB has been shown to depend on cell surface properties such as hydrophobicity and cell surface-associated molecules, including proteins, exopolysaccharides and lipoteichoic acids (Botes *et al*. 2008). Microbial cells possessing hydrophobic surfaces exhibit higher adhesive capacity compared to the hydrophilic cells (Van Loosdrecht *et al*. 1987). Our data revealed greater hydrophobicity of *L. casei* GCRL163 at the growth temperatures lower (30°C) and higher (40°C and 45°C) than the optimal temperature for growth, which suggested an improved adhesive capacity at these growth temperatures in agreement with our previous hypothesis. Concomitantly, further analysis on the cell binding capacity revealed that prolonged heat stress of *L. casei* GCRL163 at 40°C led to improved adhesion to HT-29 cells compared to 30°C. The main difference between cells cultures at 30°C and 40°C was in binding to diethyl ether, indicating that the overall charge of the cell surface could play a vital role.

This investigation has revealed new insights into the adaptation of *L. casei* GCRL163 to heat stress at the cell surface and offers more comprehensive details on the regulation of surface and secreted proteins. By comparing proteomic data from the TS, LS and ECF protein fraction, eight cytoplasmic ribosomal proteins were predicted could also be extra-cytoplasmic proteins. Intriguingly, 50S ribosomal protein L2 (RplB), 30S ribosomal protein S9 (RpsI) and 50S ribosomal protein L35 (RpmI) were predicted extracellular proteins with RpmI possessing a N-terminal signal peptide suggesting it is secreted via the Sec pathway. Tjalsma

et al. (2008) reported that one third of identified cell wall-associated proteins obtained by shaving approach in *B. subtilis* were ribosomal proteins. Also, extraribosomal moonlighting roles of some of these ribosomal proteins have been reported in bacteria (Aseev & Boni 2011). The ribosomal proteins could be involved in prolonged heat stress response as some of them including L2, L4, L11, L23, L24, L32, L33, L35 were differentially abundant at elevated temperature in *L. casei* GCRL163. Our findings also suggested reduced biofilm formation by heat stress as proteins YheA and Veg were inhibited at elevated growth temperature. This study demonstrated that adhesion of *L. casei* GCRL163 to HT-29 cells can be influenced by prolonged heat stress. Prolonged heat stress improved the adhesion of *L. casei* GCRL163 to HT-29 cells. Future advancement in scientific knowledge, including the omics especially proteomics, transcriptomics and interactomics, will provide exciting opportunities for establishing functions for proteins currently annotated as 'uncharacterized'.

Following the understanding of key surface proteins involved in adaptation of *L. casei* GCRL163 to prolonged heat stress at mid-exponential phase, we further investigated the expression of surface proteins at stationary growth phase as described in Chapter 5.

4.5 Figures and Tables

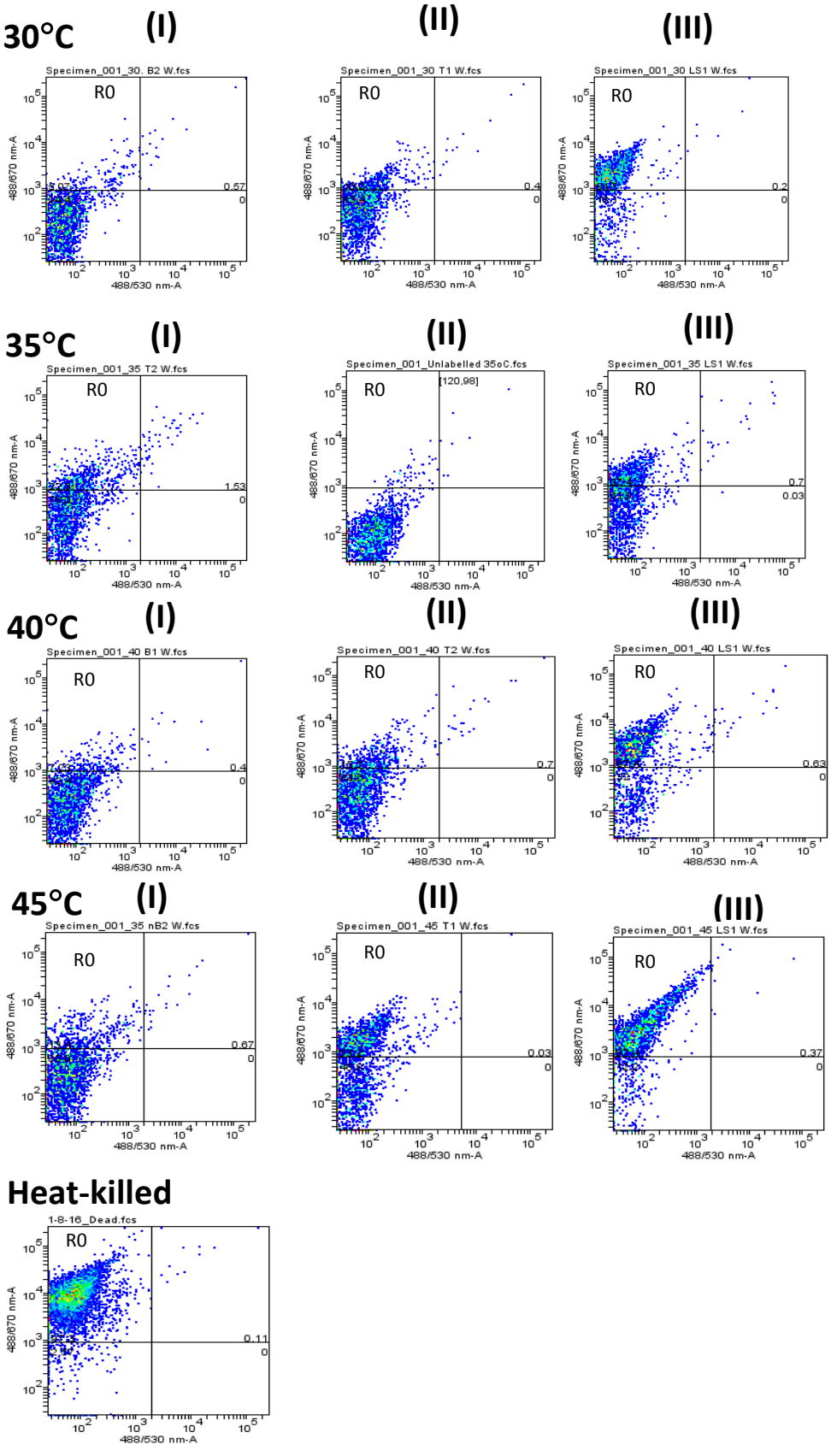


Figure 4. 1. Quadrant gating of flow cytometry dot plots demonstrating degree of cell lysis following surface protein extraction by trypsin shaving and LiCl-sucrose treatments in *L. casei* GCRL163. The gated regions denoted by R0 represent the level of cell lysis in cells grown at (A) 30°C (B) 35°C (C) 40°C (D) 45°C in (I) untreated cells, (II) cells treated with trypsin shaving, (III) cells treated with LiCl-sucrose and, (E) heat-killed cells.

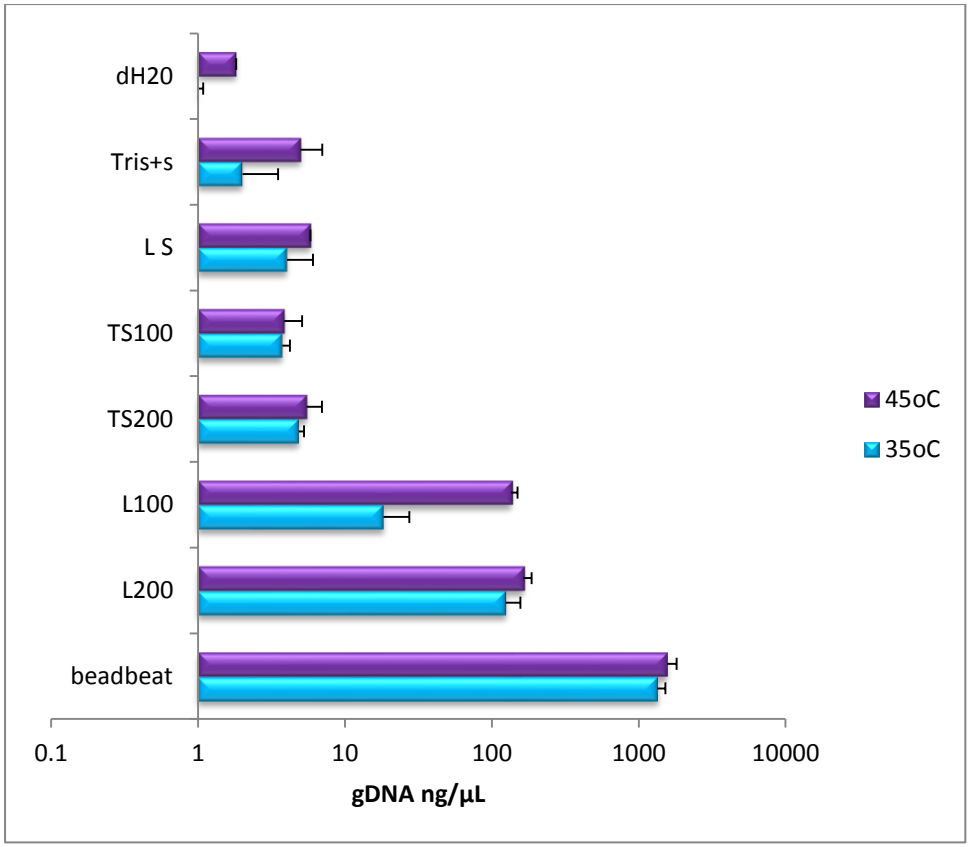
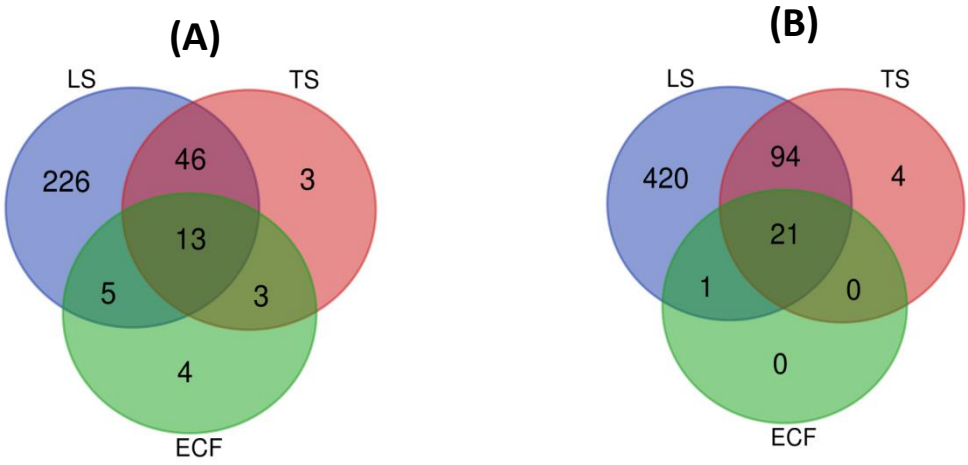


Figure 4. 2. Degree of cell lysis in *L. casei* GCRL163 monitored using DNA fragment analysis. Beadbeat: cells lysed by bead beating; L200: whole cells treated with LiCl only at 200 rpm agitation; L100: whole cells treated with LiCl only at 100 rpm agitation; TS200: whole cells trypsin shaved at 200 rpm agitation; TS100: whole cells trypsin shaved at 100 rpm agitation; LS: whole cells treated with LiCl supplemented with sucrose; Tris + s: whole cells treated with Tris buffer and sucrose; and dH20: whole cells treated distilled water.



Total predicted extra-cytoplasmic proteins 300

Total cytoplasmic-surface-associated proteins 116

Figure 4. 3. Number of cell surface proteins identified in *L. casei* GCRL163. (A) total extra-cytoplasmic proteins identified by bioinformatic subcellular prediction algorithms. (B) total cytoplasmic proteins identified as cell surface-associated proteins in at least two of the three approaches. The Venn diagram represents proteins obtained by LiCl-sucrose (LS) extraction, trypsin shaving (TS) of the whole cells and concentrated extracellular culture fluid (ECF) precipitation approaches from three technical replicates and detected with minimum of two unique peptides at FDR 5%.

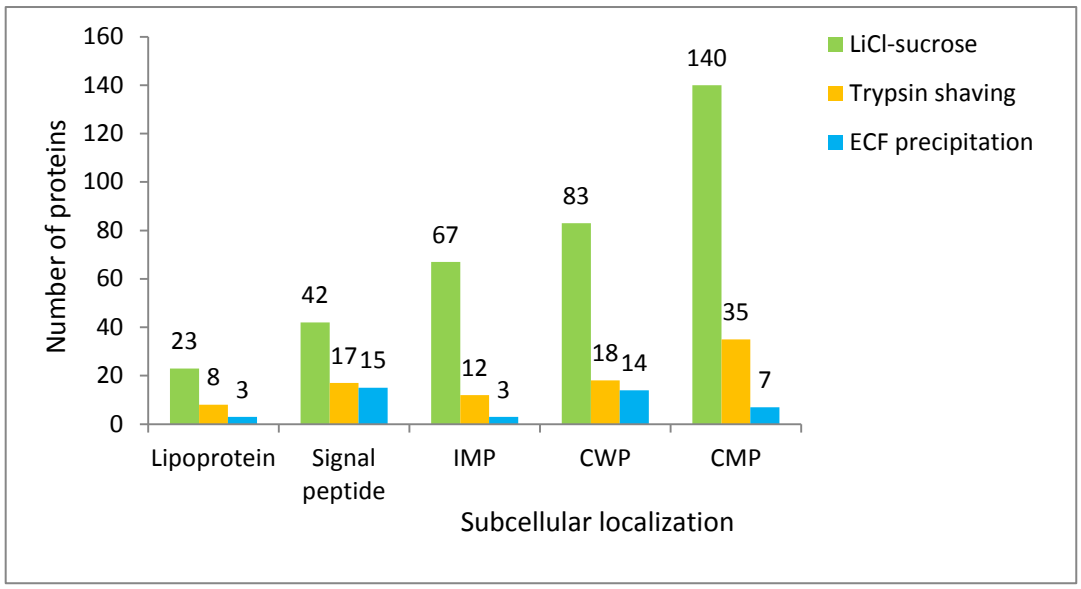


Figure 4. 4. Subcellular distribution of *L. casei* GCRL163 cell surface proteins obtained from the trypsin shaving, LiCl-sucrose and extracellular culture fluid protein fractions. The surface proteins were classified as cytoplasmic-surface-associated proteins (CTP), cell membrane-anchored proteins (CMP), cell wall-associated/extracellular proteins (CWP), proteins displaying 1 to 12 α-helical transmembrane domains and identified as integral membrane proteins (IMP). Signal peptides and lipoproteins were identified. Protein subcellular localization was assigned according to bioinformatic prediction tools: IMPs were predicted using TMHMM server 2 .0 algorithms (Krogh *et al*. 2001). The CTPs were predicted intracellular proteins with no exporting or sorting signal domain, CMPs as membrane-anchored and CWPs as proteins possessing cell wall-binding or LPXTG domains as established by Gpos-mPLoc (Shen & Chou 2009) and PSORTb 3.0.2 (Yu *et al*. 2010) algorithms. Proteins possessing N-terminal signal peptide for secretion were established using SignalP 4.1 server (Petersen *et al*. 2011) and lipoproteins predicted as lipid-anchored proteins by the Pred-Lipo server (Bagos *et al*. 2008).

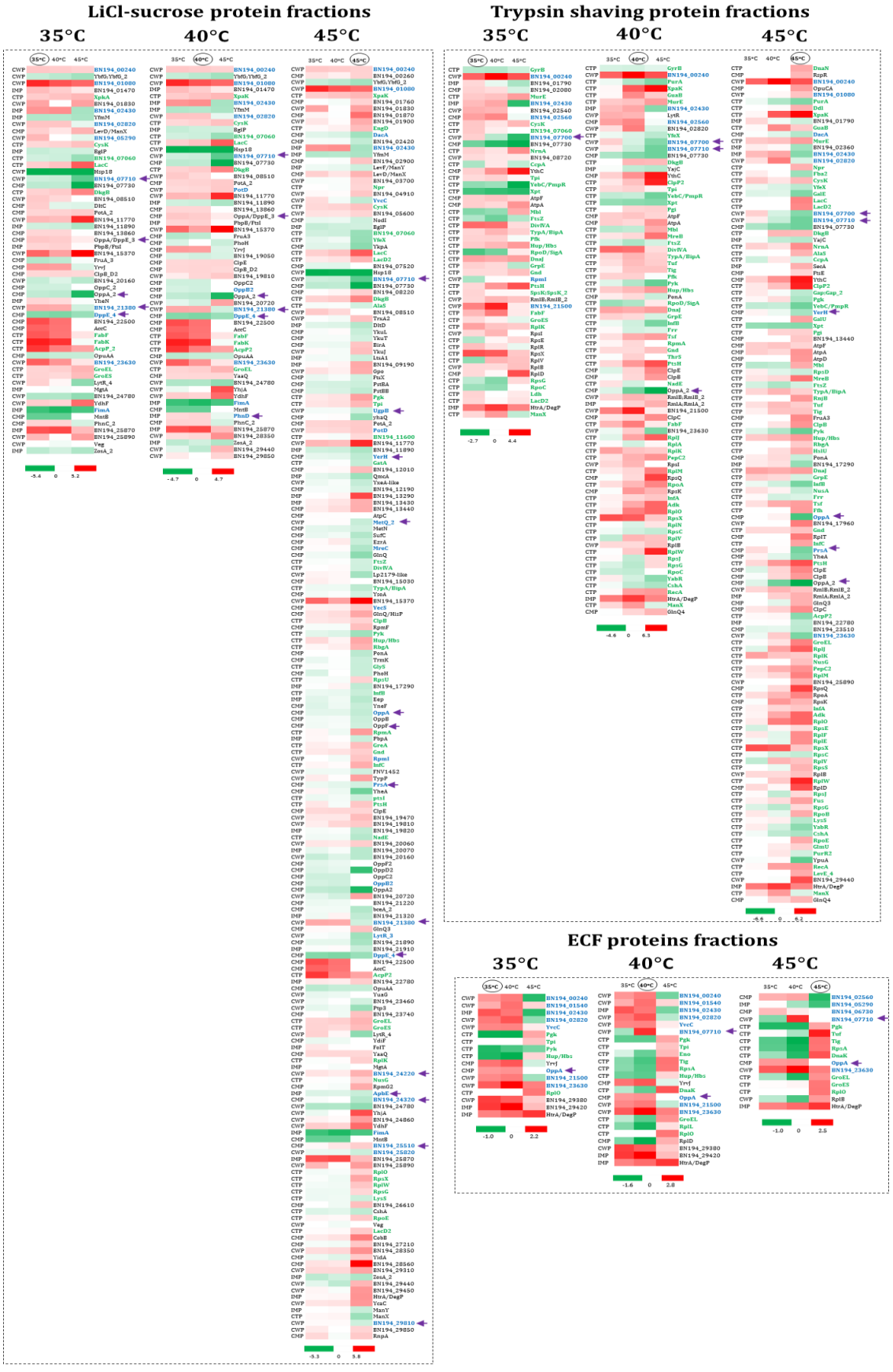

LiCl-sucrose protein fractions
35°C
40°C
45°C
Trypsin shaving protein fractions
35°C
40°C
45°C
ECF proteins fractions
35°C
40°C
45°C

Figure 4. 5. Differential expression of *L. casei* GCRL163 surface proteins under prolonged heat stress. The heat maps depict surface proteins that are differentially modulated in the LiCl-sucrose, trypsin shaving and ECF protein fractions at 35°C, 40°C and 45°C relative to expression at 30°C. LFQ values were considered significant at $\log_2$ fold threshold of ≥1.0-fold up-regulated or ≤-1.0-fold down-regulated, $p<0.05$, FDR<5% from 3 technical replicates. The scales show the differences in $\log_2$ LFQ where red, up-regulated; green, down-regulated; and white, protein detected at the 30°C control but not detected in the test growth temperature. The proteins labelled in blue represent surface proteins possessing N-terminal signal peptide for secretion while the purple arrows point to lipoproteins. Cytoplasmic proteins considered as surface proteins by detection in at least two of the three protein fractions were annotated in green colour. Protein subcellular locations were assigned according to subcellular localization prediction tools as previously described (see Figure 4. 4).

Table 4. 1. Cell wall biogenesis and related surface proteins of *L. casei* GCRL163 and their regulation following culture at different growth temperatures. Changes in the log_2-transformed LFQ values (p<0.05, FDR<5%) from three technical replicates in the trypsin shaving (TS), LiCl-sucrose (LS) and extracellular fluid (ECF) protein fractions for mid-exponential cells cultured at 35°C, 40°C and 45°C relative to 30°C are represented. Proteins not detected at 30°C but induced by prolonged heat stress (NaN) and proteins absent in the protein fraction (-) are indicated.

			LS			TS			ECF				
Gene locus	**Protein ID**	**Gene name**	**35°C**	**40°C**	**45°C**	**35°C**	**40°C**	**45°C**	**35°C**	**40°C**	**45°C**	**Protein activity or putative function**	**[kDa]**
BN194_00240	K0MRM8	BN194_00240	1.3	1.2	1.1	3.9	6.3	4.3	1.2	1.5	-0.9	Cell wall protein with CHAP domain, SibA; presumptive cell wall hydrolase	43
BN194_01390	K0MRY4[#]	*ddl*	0.4	0.5	0.5	0.0	0.2	1.9	-	-	-	D-alanine-D-alanine ligase, peptidoglycan biosynthesis	46
BN194_01540	K0MRZ8[a]	BN194_01540	-	-	-	-	-	-	0.9	1.6	0.5	WxL domain cell surface protein	28
BN194_01550	K0N1B4[a]	BN194_01550							NaN	NaN	NaN	WxL surface cell wall-binding domain protein, secreted protein	26
BN194_02140	K0MS53	dacA	-0.6	-0.8	-1.9	-0.1	-0.7	-1.9		-	-	D-alanyl-D-alanine carboxypeptidase, peptidoglycan biosynthesis	47
BN194_02160	K0N1S5[#]	*murE*	-0.2	-0.2	-0.3	1.3	1.6	2.5	-	-	-	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase	57
BN194_02360	K0N1U1	BN194_02360	-0.3	-0.1	-0.4	0.5	0.2	-2.4	-	-	-	Teichoic acid polymerase, putative	51
BN194_02420	K0N7L2	BN194_02420	-0.2	-0.2	-1.3	-	-	-	-	-	-	Glycosyltransferase family protein	29
BN194_02430	K0N547	BN194_02430	2.4	2.6	-1.8	1.7	2.2	-3.1	1.9	1.5	-0.9	Cell wall hydrolase, peptidoglycan catabolic process, lysozyme activity	101
BN194_02470	K0N7L7	BN194_02470	0.0	0.0	-0.4	-	-	-		-	-	Capsular biosynthesis protein	40
BN194_02560	K0N1V5[a]	BN194_02560	-	-	-	2.4	3.0	-0.2	0.9	1.0	-1.0	Hypothetical protein BN194_02560 , putative cell wall-associated hydrolase	36
BN194_02820	K0N7S8[a]	BN194_02820	1.3	1.3	-0.5	0.8	3.2	-1.0	1.5	1.9	-0.4	Cell wall hydrolase, NlpC/P60 domain	50
BN194_05290	K0MSU3[a]	BN194_05290	1.6	0.0	-0.6	-	-	-	0.0	-0.4	-0.9	Cell wall LPXTG motif anchor domain-containing protein	36
BN194_06750	K0N2I6[a]	BN194_06750			-		-	-	NaN	NaN	NaN	WxL domain cell surface protein	74
BN194_07350	K0N2R3[#]	*galE*	-0.3	-0.1	-0.5	-0.9	-0.8	-3.3		-	-	UDP-glucose 4-epimerase, teichoic acid decoration	36
BN194_08610	K0N361	*dltC*	1.1	-0.6	-0.7	-	-	-	-	-	-	D-alanine-poly (phosphoribitol) ligase subunit 2, lipoteichoic acid biosynthetic process	9
BN194_08620	K0N978[a]	*dltD*	-0.6	-0.7	-1.6	-	-	-	-	-	-	Undecaprenol-phosphate-poly (glycerophosphate subunit) D-alanine transfer protein, teichoic acid D-alanylation	48
BN194_08770	K0N987[a]	*pbpF*	-0.1	0.1	-0.9	-	-	-	NaN	NaN	0.0	Peptidoglycan glycosyltransferase/transpeptidase, peptidoglycan polymerization	74
BN194_09110	K0N399	*ltaS1*	-0.2	-0.2	-1.0	-	-	-	-	-	-	Exported glycerolphosphate lipoteichoic acid synthetase	78
BN194_10300	K0N3H5	*ythC*	0.6	0.7	0.7	1.2	2.7	5.8		-	-	PspC domain-containing protein, bacterial adhesin	54
BN194_12280	K0N7E3[#]	*cap4C*	-0.4	-0.3	-0.7	0.0	0.0	2.3	-	-	-	UTP-glucose-1-phosphate uridylyltransferase, probable teichoic acid decoration and exopolysaccharide biosynthesis	34
BN194_13290	K0MV00[a]	BN194_13290	0.0	0.3	4.2	-	-	-	-	-	-	Glycosyltransferase	48
BN194_14730	K0N819[a]	*murG*	-0.1	0.1	-0.7	-	-	-	-	-	-	UDP-N-acetylglucosamine-N-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol N- acetylglucosamine transferase	39
BN194_15850	K0N527	BN194_15850	0.1	0.1	0.1	-	-	-	-	-	-	DUF2140 superfamily protein, hydrolase	23
BN194_16340	K0MVK5	*yloA*	-0.1	-0.3	-0.7	-	-	-	-	-	-	Fibrinogen/fibronectin (RNA)-binding protein FpbA	64
BN194_16720	K0NAU3	*ponA*	0.0	0.0	-1.4	0.5	0.9	-1.4		-	-	Peptidoglycan glycosyltransferase, cell division; divisome complex; peptidoglycan polymerization	83
BN194_18440	K0MW71[a]	*yqgF*	0.2	0.1	-1.1	-	-	-	-	-	-	Peptidoglycan transpeptidase, peptidoglycan polymerization	77
BN194_18550	K0N5W7	*yrrL*	-0.1	0.1	-0.7	-	-	-	-	-	-	YceG-like superfamily protein, endolytic murein transglycosylase	44
BN194_19090	K0MWB2	*pbpF_2*	0.2	0.4	0.0	-	-	-	-	-	-	Peptidoglycan glycosyltransferase/transpeptidase, Penicillin-binding protein 1F	77
BN194_20060	K0N5Y7	BN194_20060	0.6	0.5	1.1	-	-	-	-	-	-	Cell-wall protein with surface anchor repeat	19

BN194_20070	K0NBE0[a]	BN194_20070	-0.5	-0.2	-1.5	-	-	-	-	-	-	DUF4330 superfamily protein, binding peptidoglycan in bacteria	19
BN194_21270	K0NBK5[a]	*yheN*	1.0	0.8	-0.1	-	-	-	-	-	-	Peptidoglycan deacetylase, Sm-PdgA family, peptidoglycan deacetylation	37
BN194_21320	K0NBK6[a]	BN194_21320	-0.3	0.3	-1.3	-	-	-	-	-	-	Peptidase C39-like family protein, glycosyl hydrolase family 2, sugar binding domain protein	40
BN194_21330;BN194_21760	K0N9X8; K0N6D6[#]	spsK; spsK_2	-0.3	-0.3	-0.1	1.0	0.2	0.2	-	-	-	dTDP-4-dehydrorhamnose reductase, also spore coat polysaccharide biosynthesis	32
BN194_21340;BN194_21770	K0NBN4; K0MWS0	*rmL; rmL_2*	-	-	-	1.0	1.9	1.5		-	-	dTDP-glucose 4,6-dehydratase; extracellular polysaccharide biosynthetic process	30
BN194_21350;BN194_21780	K0N6Q5[*]	*rmlC; rmlC_2*	-0.1	-0.2	-0.8	-	-	-	-	-	-	dTDP-4-dehydrorhamnose 3,5-epimerase, the biosynthesis of dTDP-L-rhamnose	21
BN194_21360;BN194_21790	K0N699; K0MWW4	*rmlA; rmlA_2*	-0.1	-0.2	0.1	0.0	1.2	2.0		-	-	Extracellular polysaccharide biosynthetic process	35
BN194_21390	K0MWS4[a]	BN194_21390	0.2	0.1	-0.5					-	-	Polyprenyl glycosylphosphotransferase, teichoic acid biosynthesis	54
BN194_21500	K0N6S2	BN194_21500	0.6	0.6	0.1	3.1	5.5	0.5	1.3	1.5	-0.4	Cell wall-associated hydrolase, NlpC/P60 family	43
BN194_21890	K0MWX1	BN194_21890	-0.5	-0.5	-1.8					-	-	Putative exopolysaccharide biosynthesis protein (Mrp, Chromosome partitioning ATPase)	30
BN194_22780	K0NA48	BN194_22780	0.5	0.4	1.1	0.0	0.1	-1.6		-	-	Sortase family protein	30
BN194_23630	K0NA78	BN194_23630	3.7	2.5	0.6	-0.4	1.3	-3.4	1.8	2.8	1.4	Hypothetical protein, putative cell wall-associated hydrolase	38
BN194_26940	K0MY10[#]	*glmU*	-0.1	-0.1	-0.8	0.0	0.5	1.1		-	-	Glucosamine-1-phosphate N-acetyltransferase / UDP-N-acetylglucosamine pyrophosphorylase	50
Cytokinesis													
BN194_02040	K0MS45	*soj*	0.2	0.2	0.4	-	-	-	-	-	-	Chromosome partitioning ATP-binding protein	28
BN194_10190	K0MTZ2	*ftsE*	-0.1	-0.1	0.3	0.0	0.0	1.1	-	-	-	Cell division ATP-binding protein	26
BN194_10200	K0N3G0	*ftsX*	-0.4	-0.2	-0.9	0.5	-0.1	0.0	-	-	-	Cell division protein	33
BN194_13650	K0N4K5[#]	*mbl*	-0.2	-0.2	-0.8	-1.8	-1.6	2.1	-	-	-	Cell-shape determining protein	35
BN194_14460	K0N4J2	*ezrA*	0.1	0.5	-1.1	-	-	-	-	-	-	Septation ring formation regulator	64
BN194_14550	K0N4R7[#]	*mreB*	-0.3	-0.4	-0.9	0.6	1.0	3.4	-	-	-	Rod shape-determining protein	35
BN194_14560	K0N4K0	*mreC*	0.1	0.1	-1.6	-	-	-	-	-	-	Rod shape-determining protein	31
BN194_14590	K0MV70	*minD*	0.1	-0.1	-0.2	-	-	-	-	-	-	Septum site-determining protein	29
BN194_14700	K0N4S9[a]	*pbpB*	1.0	1.1	0.1	-	-	-	-	-	-	Septal peptidoglycan transpeptidase, divisome complex	77
BN194_14750	K0N4T3	*ftsA*	0.0	0.0	-0.5	-	-	-	-	-	-	Cell division protein	48
BN194_14760	K0N4L6[#]	*ftsZ*	-0.1	0.0	-1.0	-1.1	-2.1	-1.1	-	-	-	cell division protein FtsZ, cell shape directing complex	45
BN194_14800	K0N4T8[#]	*divIVA*	0.0	0.1	-1.0	3.7	3.6	0.9		-	-	Septum site-determining protein	28
BN194_17200	K0N5G4[a]	*yrvJ*	2.3	2.2	-0.7	-	-	-	1.6	1.9	-0.2	N-acetylmuramoyl-L-alanine amidase, septum splitting	49

[a] Proteins not detected in cell-free extracts

*****Protein encoded by *rmlC* is not a predicted surface protein but one of the four precursors in the dTDP-L-rhamnose pathway for exopolysaccharide synthesis

[#]Cytoplasmic proteins considered as surface proteins by detection in at least two of the three protein fractions.

Table 4. 2. Cell surface hydrophobicity of *L. casei* GCRL163 under prolonged heat stress

Temperature (°C)	**Hexadecane (%)**	**Chloroform (%)**	**Diethyl ether (%)**
30	27.1 ± 0.26	72.1 ± 0.85	20.5 ± 2.01
35	16.3 ± 1.08	65.6 ± 2.47	21.7 ± 2.55
40	28.6 ± 0.64	73.0 ± 1.16	38.7 ± 0.40
45	24.7 ± 0.06	69.1 ± 1.28	31.0 ± 1.36

Triplicate experiments ($\overline{x} \pm S$, $P<0.05$)

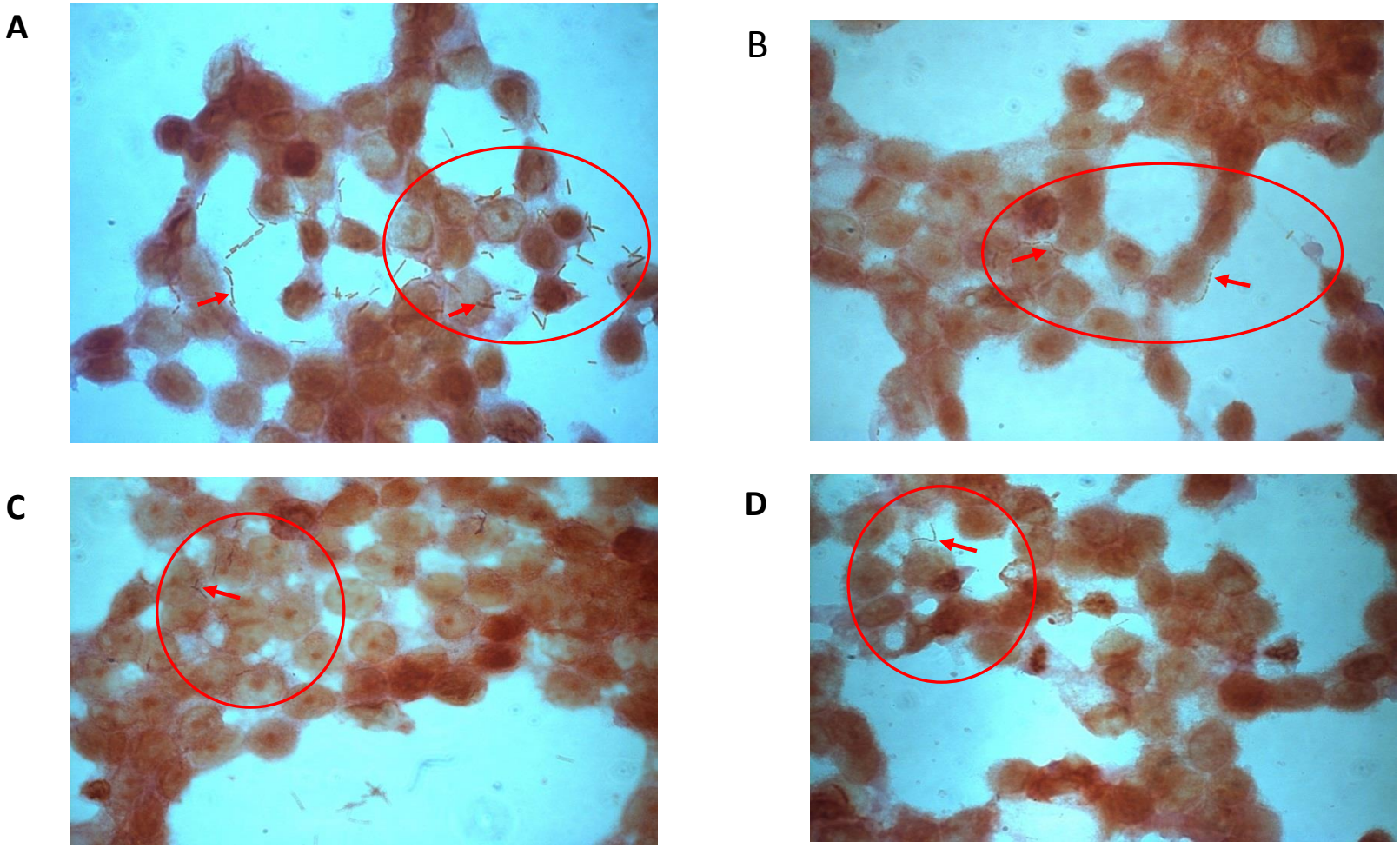


Figure 4. 6. Adhesion of Gram-stained *Lactobacillus* strains to HT-29 cells. (A) *L. helveticus* K1 (B) *L. rhamnosus* NBRC3425 (C) *L. casei* GCRL163 and (D) *L. paracasei* 7K07A2. Images from approximately 60% confluence regions were examined to allow for clarity (x 100 magnification).

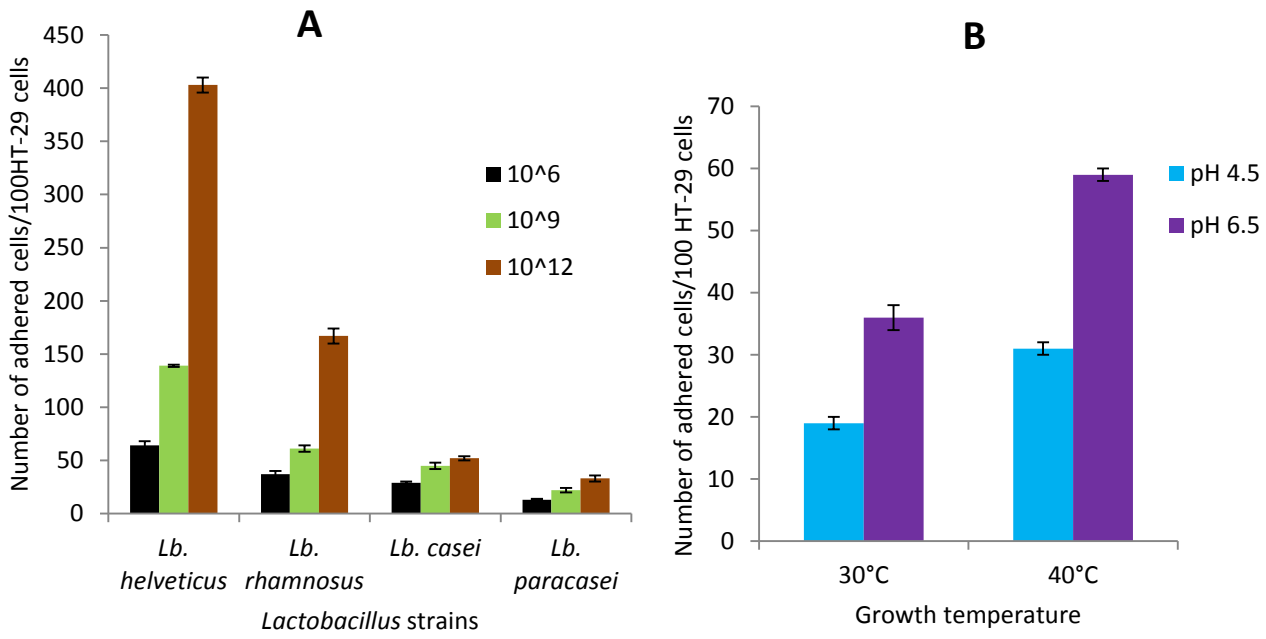


Figure 4. 7. Adhesion of the *Lactobacillus* spp. cells to HT-29 cells. (A) Different *Lactobacillus* strains applied to HT-29 cells at different concentrations and (B) *L. casei* GCRL163 cells cultured at different temperatures and pH (10^{12} CFU/mL applied to HT-29 cells).

CHAPTER 5

COMPARATIVE PROTEOMICS OF *LACTOBACILLUS CASEI* GCRL163 SURFACE PROTEINS AT THE MID-EXPONENTIAL AND STATIONARY GROWTH PHASES

5.1 Abstract

Bacterial cell surfaces are vital structures through which contact is made with the external environment, including sensing adverse conditions. Understanding the expression of proteins associated with the cell surface at different growth phases can provide an in-depth insight into how bacteria manipulate their physiological processes to adapt under different environmental conditions. We applied label-free quantitative proteomics to investigate *L. casei* GCRL163 surface proteins extracted by trypsin shaving (TS) and LiCl-sucrose (LS) at mid-exponential and stationary growth phases and also profiled the secreted extracellular culture fluid (ECF) proteins. Our findings revealed an increased expression of some cell wall hydrolases, including BN194_23630 and SLAP domain-containing NlpC/P60 protein BN194_02820, and putative autolysins, including YrvJ and glycoside-hydrolase B194_02820, at stationary phase, suggesting induced cell wall hydrolysis, peptidoglycan autolysis and exopolysaccharide degradation in stationary phase. Proteins involved in sugar and peptide uptake, including oligopeptide ABC-type transporters OppA, OppA_2 and OppD_2, and phosphotransferase system proteins FruA_3 and BglP were more abundant, with GlnA and CysK induced at stationary growth phase in different protein fractions. Transcriptional regulator LytR, involved in sensory transduction and cell wall metabolism regulation, and proteins associated with cell surface structure synthesis, including exopolysaccharides such as RmlB, RmlB_2, SpsK and SpsK _2, were more abundantly expressed at stationary phase. The abundance of several proteins linked to probiotic

functionality, including moonlighting proteins (Gap, Fab_2), Tig and cell wall hydrolase NlpC/P60 protein BN194_02820, suggests that host-cell interactions would be heightened at stationary growth phase. These findings establish the growth-phase dependent change in the surface sub-proteome of *L. casei* GCRL163 that possibly promotes cell adaptation and enhanced functionality.

Keywords: Proteomics, growth phases, *Lactobacillus casei*, cell surface proteins, Lithium chloride, trypsin shaving, secreted proteins

5.2 Introduction

Lactobacillus species are Gram-positive, aciduric or acidophilic, non-sporing, aerotolerant, anaerobic lactic acid bacteria (LAB), which are important in food microbiology and human nutrition for their roles in probiotic formulations and as dairy starters (Parvez *et al*. 2006). They are dominant in fermented dairy food products such as yogurts and cheese, which are used commonly as delivery systems to the host targets (Govender *et al*. 2014). They also contribute to cheese ripening and flavour development as non-starter lactic acid bacteria (NSLAB) (Fox *et al*. 1998; Smit *et al*. 2005). One of the dominant NSLAB species in cheese is *L. casei* (De Angelis *et al*. 2001). During Cheddar cheese production, NSLAB undergo different growth phases as nutrient availability changes during ripening, during which the cell densities increase (Ganesan *et al*. 2014). Several adaptive mechanisms are involved in cell adaptation during the growth phases (Laakso *et al*. 2011). In the proteomic and transcriptomic growth phase studies of *L. rhamnosus* GG, grown in whey medium using strictly controlled bioreactor systems, a shift from glucose fermentation to galactose utilization accompanied by homolactic fermentation changing to mixed-acid fermentation was noted at stationary growth phase (Laakso *et al*. 2011). The sustenance of bacterial

growth, in cheese characterized by nutritional limitation, involves metabolizing peptides, amino acids and sugars derived from casein hydrolysis and peptidoglycan degradation (Peterson & Marshall 1990). Cells at stationary growth phase induce proteins needed to scavenge for nutrients and survive the nutritional limitations (Bernhardt *et al*. 2003). Moreover, proteins involved in stress responses, cell wall structures and metabolic pathways for generating sufficient energy were induced in *L. plantarum* WCFS1 during stationary growth phase (Cohen *et al*. 2006).

Most of the research on the cell surface sub-proteome have considered *Lactobacillus* species that possess surface (S)-layers (Kelly *et al*. 2005; Klotz *et al*. 2017). The S-layers are outermost proteinaceous cell envelope structures, constituted by several identical subunits which form a semi-porous, lattice-like layer around the cell surface and are present in several lactobacilli but not all species (Hynönen & Palva 2013). The S-layers form a vital structure that anchored secreted proteins to the cell surface in *L. acidophilus* homology group and are absent in *L. casei,* which lack *slp* genes (Johnson *et al*. 2016). In the S-layer-containing bacteria, responses in the surface sub-proteome at different growth phases differ between species. For instance, a number of highly abundant moonlighting proteins including the cytoplasmic proteins enolase, trigger factor, fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase, were repressed in *L. acidophilus* cell surface at stationary growth phase (Klotz *et al*. 2017), whereas these non-classically secreted proteins were up-regulated during stationary growth phase in *L. salivarius* cell surface (Kelly *et al*. 2005). The cell surface molecules, especially the surface sub-proteome, are important in NSLAB as they participate in various physiological functions including cell survival and mediation of host-bacterial interactions (Polak-Berecka *et al*. 2014). Furthermore, the NSLAB can also secrete some extracellular proteins which play significant roles in the cell

adaptation, immunomodulation and interactions with host mucosal and intestinal cells (Sanchez *et al*. 2008). Recent advancement in proteomic technologies has offered immense opportunities for the identification and quantification of the surface sub-proteome under various environmental and physiological conditions (Champomier-Vergès *et al*. 2002).

In the current study, we have applied gel-free proteomics to investigate the cell surface and extracellular culture fluid (ECF) protein fractions of Cheddar-cheese isolate *L. casei* GCRL163 at mid-exponential and stationary growth phases. Several proteins were differentially regulated in the LS and TS fractions at the two growth phases while many showed varied abundances. Proteins, including SLAP domain-containing NlpC/P60 protein BN194_02820 and several cytoplasmic proteins associated with moonlight functions, which were linked to probiotic functionality in *Lactobacillus* species, demonstrated high abundances at stationary growth phase.

5.3 Results

5.3.1 Quantitative proteomics of the protein fractions reveals the impact of growth phases on cell surface protein expression

This study reports the comparative analysis of the proteins in the outer sub-proteome fractions obtained at mid-exponential and stationary growth phase cultures of *L. casei* GCRL163, grown anaerobically at 30°C in bioreactors under controlled growth conditions and pH of 6.5. Trypsin shaving (TS) and LS treatments were used to detect surface-exposed and surface-located proteins respectively while the extracellular culture fluid (ECF) proteins (proteins released into the growth media) were precipitated and concentrated using trichloroacetic acid-acetone. The experimental conditions were selected to minimize cell lysis

during protein extraction with LS (Chapter 4) and to provide a platform for determining changes in surface protein expression due to transition into stationary phase in the absence of thermal stress and minimising stress caused by low pH (notwithstanding stress caused by accumulation of lactate and other fermentation end-products in stationary phase). Protein datasets obtained using high-throughput nano-liquid chromatography and high resolution tandem mass spectrometry (nanoLC-MS/MS) were filtered by excluding proteins detected with <2 matching peptides and identified by reverse database matches, only by site or as potential contaminants. Using these criteria, LS extracts yielded 887 and 376 filtered proteins at mid-exponential and stationary growth phases respectively (Supplementary Table 5. 1). Of these, 362 proteins were found at both growth phases (Figure 5. 1 and Supplementary Table 5. 2). In TS fractions, 128 and 202 filtered proteins were detected at mid-exponential and stationary phases respectively. Of these, 93 proteins were detected at both growth phases. Moreover, a total of 49 and 88 proteins were detected in the ECF protein fractions at mid-exponential and stationary growth phases respectively, of which 39 proteins were found at the two growth phases. The datasets therefore provided the basis for determining the change in expression of proteins common across the two growth phases together with identifying growth phase-specific surface protein expression.

However, examination of the raw data indicated that direct comparison of LFQ at the different cell harvest points was problematic, due to different total LFQ values seen between datasets and lack of internal (standardizing against a protein known not to change between growth phases) or external (an added non-*Lactobacillus* protein that would detect LFQ variations due to altered operating conditions) standardization (Li *et al.* 2009). The cells were cultured in two separate experiments, albeit under the same controlled growth conditions, and analysis of all extracts from mid-exponential cells were processed in parallel, enabling

comparisons of relative abundance to be made across the different fractions (Chapter 4), and similarly for the stationary phase cells which were processed independently of the mid-exponential samples. This potentially introduced variables, including ostensible LFQ changes at the different growth phases due to variations in efficiency of protein extraction and trypsinisation between cell batches, measurement of protein concentrations for application to nanoLC/MS/MS and variation in LC conditions between runs. It was clear when analysing the data for condition-specific proteins that many of these were low to very low abundance, so that their detection or lack thereof, was highly dependent on all of the aforementioned factors. This was particularly evident in the LS fractions, which contained a background of low abundance proteins present due to lysis. Furthermore, it was clear that differences in the LFQ values in stationary phase for the majority of individual proteins in, for example, the LS were lower when compared with mid-exponential LFQ, indicating a down-shift in overall LFQ. Consequently, to identify proteins which were significantly altered in relative abundance between the growth phases, for each fraction type we considered the average difference in normalized LFQ value for all of the proteins in common across the growth phases and then determined the outliers from this average as an indicator of proteins which were differentially expressed at a particular growth phase. This is demonstrated in Figure 5. 2, for a selection of proteins from the LS fractions.

5.3.2 Differentially expressed proteins at mid-exponential and stationary growth phases are associated with different cellular functions.

The majority of differentially abundant proteins detected at the two growth phases were associated with several functional classes, based on functional annotations of Clusters of Orthologous Group (COG) (Tatusov *et al*. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000). Several of these proteins were differentially

expressed during transition into stationary growth phase relative to mid-exponential growth phase in different protein fractions from *L. casei* GCRL163 cell surface (Figure 5. 3). The proteomic analysis of the LS fraction revealed that the majority of the detected proteins associated with cell wall biogenesis were repressed in cells at stationary growth phase. However, a few of this group of proteins were over-expressed at the stationary phase in the LS, TS and ECF fractions. Notably, cell wall hydrolase BN194_23630, SLAP domain-containing NlpC/P60 protein BN194_02820 and putative autolysins including cell wall hydrolase BN194_02430 and N-acetylmuramoyl-L-alanine amidase YrvJ, were up-regulated in different fractions of the LS or ECF at stationary growth phase. Proteins involved in the synthesis of precursors for exopolysaccharides, including isoforms RmlB, RmlB_2 and SpsK, SpsK _2, showed increased expression in the TS fractions.

Of the proteins associated with cytokinesis detected in LS fractions, septum site-determining protein DivIVA and cell cycle initiation protein GpsB were up-regulated while others, including FtsA, FtsE and FtsZ, were repressed at stationary growth phase relative to mid-exponential growth phase. MreB and FtsZ in the TS fractions and YrvJ in the ECF fractions showed moderate upregulation.

The ATP synthase subunits AtpA, AtpD and AtpF were relatively more abundant in the TS fraction and other membrane bioenergetics-associated proteins, such as exopolyphosphatase Ppx and YkuN_2, were moderately increased in the LS fractions at stationary growth phase. We detected ArlR, PhoP and universal stress protein UspA superfamily BN194_22870 to be moderately repressed in the LS fractions at stationary growth phase. Cofactor NH_3-dependent NAD^+ synthetase NadE was over-expressed in the

TS and repressed in the LS fractions while others, including Fhs, GarB, MetK, SurC and BN194_03700, showed decreased expression in the LS fractions at stationary growth phase.

We observed repressed expression of most detected protein associated with amino acid metabolism, including the peptidases PepC_2, PepO_2, and PepV and several others, in the LS fractions at stationary growth phase (see Figure 5. 3). However, CysK was up-regulated in the LS and TS fractions and MetA in the LS fractions. The majority of proteins, detected to be associated with nucleic acid and nucleotide metabolism, were repressed in the LS fraction at stationary growth phase, including PyrG and PyrH, involved in pyrimidine metabolism, and PurA, involved in purine metabolism. However, PyrF, Xpt, GuaB and Ndk showed increased expression in the LS fractions and also GuaB in the TS fractions at stationary growth phase. Most of the proteins associated with DNA replication, including GyrA, GyrB, DnaN and LigA, or DNA repair and recombination, including ExoA, were down-regulated in the LS fractions at stationary growth phase. However, Ssb and Hup were moderately over-expressed at stationary growth phase in the LS fractions. Among the detected proteins associated with tRNA/ribosome assembly and processing, YabR and EbsC were moderately over-expressed in the LS fractions and Hpf in the TS fractions at stationary growth phase. Others were repressed, including Era, QueA, RluB, Rnc, Rnr and YbxB, in the LS fractions at stationary growth phase. The majority of proteins associated with tRNA aminoacyl synthesis, detected in the LS fractions were repressed, notably, ArgS, AspS, GatA, HisS, and ProS at stationary growth phase. However, ThrS, GatA and GatC were over-expressed in the TS fractions at the growth phase.

Furthermore, proteins involved in the transcriptional regulation were detected mostly in the LS protein fractions and showed repressed expression (see Figure 5. 3). However,

GntR family transcriptional regulator YvoA was over-expressed in the LS fractions while catabolite control protein A (CcpA) and LytR family cell envelope-related function transcriptional attenuator LytR were also up-regulated in the TS fractions at stationary growth phase. Proteins Csp and CspLA associated with transcription were over-expressed in the LS fractions with Csp also up-regulated in the ECF fractions at stationary growth phase. Proteins associated with protein translation, including Tsf and Fus, were up-regulated in the TS and ECF fractions while Tuf and EngD showed increased expression in the TS fractions at stationary growth phase. However, all the detected proteins in the LS fractions involved in the protein translation were repressed, including Efp, Fus, Tsf, Tuf, TypA, EngD and Map, at stationary growth phase. Most of the ribosomal proteins detected in the LS fractions were also down-regulated at stationary growth phase. However, upregulation of 30S ribosomal protein RpsA was detected in the LS fractions while 30S ribosomal proteins RpsM, RpsB, RpsH and 50S ribosomal proteins RplK, RpsQ, RplJ, RplE were induced in the TS and RplK in the ECF fractions at stationary growth phase.

Proteins associated with carbohydrate metabolism and glycolytic/intermediary pathways were mostly down-regulated in the LS fractions at stationary growth phase. However, some of them were seen up-regulated in the TS fractions, including LacD2_2 and Ldh. Fab_2 and Gap in the glycolytic pathway and XpaK and Gnd in the pentose phosphate pathway at stationary growth phase. We also detected AcpP, AcpP_2 and FabZ, involved in lipid metabolism, to show greatly enhanced expression at stationary growth phase in the LS fraction. Of the proteins involved in protein folding and turnover, GrpE and ClpB_2 were up-regulated in both LS and TS fractions while overexpression of Tig, HslU and ClpP_2 were detected only in the TS fraction and HtrA in the ECF fraction. Phosphocarrier protein HPr, involved in the PTS, was also over-expressed in the LS fraction at stationary growth phase.

Of the proteins associated with the ABC-type transporter systems, ABC transporter substrate-binding protein BN194_07700 was over-expressed in the LS fraction and OppA_2 in the TS fraction. OppA and BN194_07710 were also up-regulated in the ECF fraction. Cadmium efflux system accessory protein CadC was over-expressed at stationary growth phase in the LS fraction.

5.3.3 Proteins unique to the mid-exponential and stationary growth phases

To differentiate between proteins which were low abundance and likely to be detected due to cell lysis, we considered proteins with a $\log_2$-transformed LFQ of >23 as significantly and uniquely expressed at the different growth phases. Several of these proteins were also predicted, using various subcellular localization prediction servers (Emanuelsson *et al*. 2000), to be localized at different subcellular locations in the cells and they were detected in LS, TS and ECF fractions (Table 5. 1). The expression of these proteins in high abundance may signify their importance in the adaptive mechanisms of the cells at different growth phases. Among these highly abundant specific proteins, GlmS, involved in early peptidoglycan biosynthesis, was detected at stationary growth phase in the TS fraction while RmlB, involved in rhamnose-containing exopolysaccharide synthesis was abundant at mid-exponential growth phase in the LS fraction. Cell-shape determining protein Mbl, associated with cytokinesis, was also abundantly expressed in the LS fractions at mid-exponential growth phase.

Of the proteins associated with amino acid metabolism which were specific to the growth phases, PepN and PepF were abundantly expressed at mid-exponential growth phase in the LS fraction while PepC_2 and GlnA were highly abundant at stationary growth phase in the TS fractions. PurC and PurH, involved in purine biosynthesis in the nucleic acid and

nucleotide metabolism, were detected to be abundantly expressed at stationary growth phase in the TS fraction. YebC/PmpR family BN194_11600 and TetR family transcriptional regulator BN194_12190 were detected to be abundantly expressed at mid-exponential growth phase in the TS and LS protein fractions respectively. RNA chaperone Csp, associated with transcription, was found to be abundantly expressed in the TS fraction at mid-exponential growth phase. RNA binding protein, S1-like superfamily YabR, involved in tRNA/Ribosome assembly and processing was abundant in the TS fractions at mid-exponential growth phase. Specific proteins at mid-exponential growth phases, associated with tRNA charging in the tRNA aminoacyl synthesis, were detected in the LS protein fraction and included AlaS, GatB, ValS, IleS, GlyS and SerS. We also detected some ribosomal proteins which were specific to the growth phases in the protein fractions. Notably, 30S ribosomal proteins RpsR and RpsZ, and 50S ribosomal protein RpmG were abundantly expressed at mid-exponential and RpsI at stationary growth phase in the LS fractions. Furthermore, 30S ribosomal protein RpsK at mid-exponential growth phase and 50S ribosomal proteins RplS, RplO and RplW at stationary growth phase were abundantly expressed in the TS fractions.

In the phosphotransferase systems (PTS), integral membrane protein BglP, involved in glucose uptake and cell membrane-anchored FruA_3, a fructose/mannose/sorbose family transporter, were found in LS fractions to show high abundance specifically at mid-exponential growth phase. Furthermore, oligopeptide ABC-type transporter ATP-binding protein OppD_2 was highly abundant in the LS at mid-exponential and stationary growth phases in the TS fractions. Other highly abundant proteins, involved in the ABC transport system at mid-exponential growth phase include OppF and YfiB in the LS and ABC-type transporter, substrate-binding protein BN194_07700 in the TS protein fraction.

Of the proteins involved in protein folding and turnover, α-crystallin domain heat shock protein Hsp18, associated with protein disaggregation during rapid growth, and FtsH, involved in membrane protein assembly and recycling, and ClpX, demonstrated abundant expression at mid-exponential growth phase in the LS fraction. Also, AdhE, associated with carbohydrate metabolism, was detected in high abundance at mid-exponential growth in the LS fraction. In the TS fraction, Pgi was abundantly expressed only at stationary growth phase. Other proteins specific to mid-exponential growth phase include YpuA in the TS, and YbaB/EbfC DNA-binding family protein BN194_23980 and RpoC in the LS fractions. These proteomic data suggested that several proteins involved in stress responses were induced during growth at stationary phase.

5.4 Discussion

The cell surface properties including surface proteins of *Lactobacillus* population employed in probiotic formulations and dairy starters often experience stress-induced alterations during cell growth (Deepika *et al*. 2009). These growth-dependent changes impact on the physiological functions and survival of the dairy strains (Laakso *et al*. 2011). The physiological state of bacteria is considered one of the most important factors that determine cellular protein profiles or expressions and hence their functional characteristics (Phan-Thanh & Mahouin 1999). Therefore, understanding key changes in the surface sub-proteome at different growth phases in dairy strains can shed more light on complex network of growth-dependent physiological and cellular processes that facilitate *Lactobacillus* adaptation and improved probiotic strain selection.

The availability of the exogenous amino acids and peptides, especially in the nutritionally limited stationary growth phase, involves a network of hydrolytic and

proteolytic systems, including proteinases which degrade proteins to oligopeptides, oligopeptide ABC-transporter systems involved in peptides uptakes, and peptidases which are associated with the release of the peptides and amino acids from the oligopeptides (Law & Haandrikman 1997; Liu *et al*. 2010). In the current study, the increased expression of cell wall hydrolases, including BN194_23630 and SLAP domain-containing NlpC/P60 protein BN194_02820, and putative autolysins, including YrvJ and protein B194_02820 with a peptidoglycan autolysin glycoside-hydrolase-lysozyme domain, at stationary growth phase, could indicate induced cell wall hydrolysis, peptidoglycan autolysis and exopolysaccharide degradation, which released amino acid and nucleotide sugar derivatives from the cells and growth media for bacterial nitrogen sources. Although most of the peptidases, including PepC_2, PepO_2, and PepV, demonstrated low abundances at stationary growth phase, GlnA was specifically abundant while CysK and MetA were up-regulated in LS or TS fractions, possibly to generate exogenous nitrogen sources. Although, most detected proteins associated with purine (e.g. PurA) and pyrimidine (e.g. PyrF and PyrH) syntheses were down-regulated in the LS fractions, high abundant expression of PurC and PurH, involved in purine synthesis, was observed at in the TS fraction and some proteins involved in nucleic acid and nucleotide metabolism, including PyrF, Xpt, GuaB and Ndk were over-expressed mostly in the LS fraction at stationary growth phase. Intriguingly, the high abundance of oligopeptide ABC-type transporters OppA_2 and OppD_2 in the TS fraction and OppA in the ECF, and FruA_3 and BglP of the PTS is consistent with the high abundant expression of the cell wall hydrolases and autolysins as well as GlnA and CysK in generating amino acids, peptides and sugars which are vital for sustaining growth at stationary growth phase.

Furthermore, proteins associated with ATP synthesis, including ATP synthases AtpA, AtpD and AtpF were over-expressed at stationary growth phase in the TS fraction. ATP

synthases have been associated with intracellular pH homeostasis through proton extrusion (Cotter & Hill 2003). The enhanced expression of ATP synthases could be in response to cellular accumulation of protons or the need for an increased ATP generation at stationary growth phase. However, the bacterial cultures were grown under controlled conditions in bioreactors with online addition of alkaline to maintain the pH, thereby controlling the effect of proton accumulation. The ATP generated could be used for the synthesis of cellular macromolecules, thereby enhancing cell structure maintenance (Konings *et al*. 1997). An increased expression of Gap and Fab_2 and abundantly expressed Pgi, involved in glycolysis, in the TS fraction suggest an enhanced ATP synthesis. The glycolytic enzymes are among the cytoplasmic proteins which can be present at the cell surface due to secondary roles as moonlighting proteins, contributing to host-bacterial interactions (Wang *et al*. 2013). Others, including DNA-binding protein Hup, involved in adaptation to stress conditions (Whiteford *et al*. 2011) and laminin-binding adhesion in *L. rhamnosus* FSMM2 (Aryantini *et al*. 2017), and trigger factor Tig, associated with pathogen exclusion in *L. fermentum* RC-14 (Heinemann *et al*. 2000) were over-expressed in the TS fraction at stationary growth phase. These findings suggest that improved cell adhesion correlates with stationary growth phase.

Several other stress-related proteins were abundantly expressed at stationary growth phase in the *L. casei* GCRL163 cell surface, especially the proteolytic systems for protein folding and maintenance of irreparably injured proteins. These include GrpE, Tig, HslU HtrA and proteases ClpB_2 and ClpP_2 which were variously up-regulated in the protein fractions. The upregulation of proteins such as isoforms RmlB, RmlB_2 and SpsK, SpsK, associated with surface structure synthesis including exopolysaccharides could indicate that these proteins were recruited for maintaining cell envelope at stationary phase in *L. casei* GCRL163. While proteins associated with protein translation were markedly repressed in the

LS fraction at stationary growth phase, some of these proteins including EngD, Tsf, Tuf and Fus were moderately up-regulated in the TS fraction. Moreover, proteins associated with tRNA aminoacyl synthesis, including GatA and GatC, which were repressed in the LS protein fraction, became over-expressed in the TS fraction. These findings suggest a subcellular reshuffling of protein locations during growth resulting in most of these proteins becoming surface-exposed or surface-located, thereby accessible to either LS or TS treatment.

This study has provided further insight into the physiological changes occurring in *L. casei* GCRL163 at mid-exponential and stationary growth phases using proteomics. Although proteins vital for cellular adaptation were detected, several of the detected proteins in the cell surface fractions could not be identified. We assigned putative identity to several uncharacterized proteins using protein homologs through UniProt (Consortium 2016) and protein sequence analyses by BLASTN through Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000) and the National Centre for Biotechnology Information (NCBI) (Coordinators 2017). However, the proteomic data showed several relatively up-regulated uncharacterized proteins at stationary growth phase in the LS fraction, many of which were predicted cell wall-anchored proteins. These proteins may be important for cellular adaptation and functions and a further improvement in proteomic and bioinformatic tools will assist in future protein identification.

Our findings in Chapter 3 to 5 have demonstrated that a variety of cytoplasmic and surface proteins, associated with different processes, are involved in adaptation of *L. casei* GCRL163 to prolonged heat stress. By performing a forensic analysis, key proteins involved

in the regulation of prolonged heat stress in *L. casei* GCRL163 were identified and the expression described in Chapter 6.

5.5 Figures and Tables

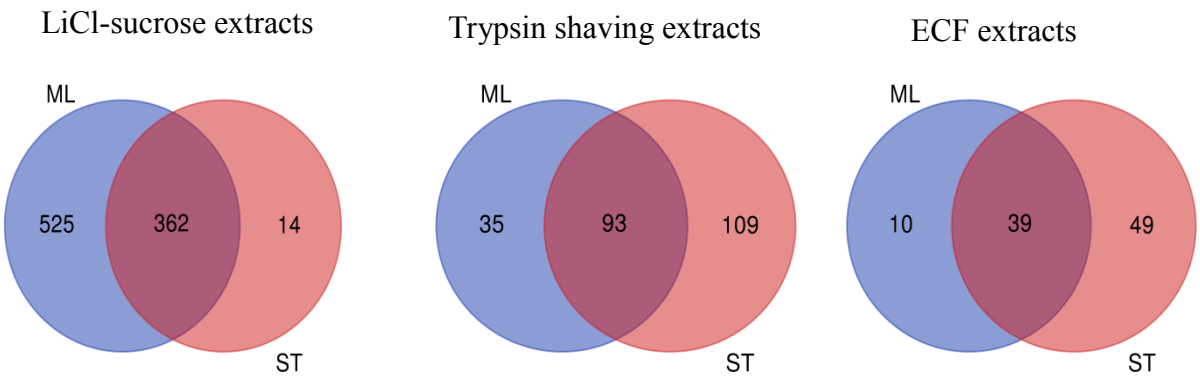


Figure 5. 1. Distribution of proteins detected in different protein fractions at mid-exponential (ML) and stationary (ST) growth phases in *L. casei* GCRL163 cell surface. The Venn diagrams represent proteins obtained by LiCl-sucrose treatment and trypsin shaving of the whole cells and proteins released into the growth medium or extracellular culture fluid (ECF) from three technical replicates and detected with minimum of two unique peptides at FDR 1%.

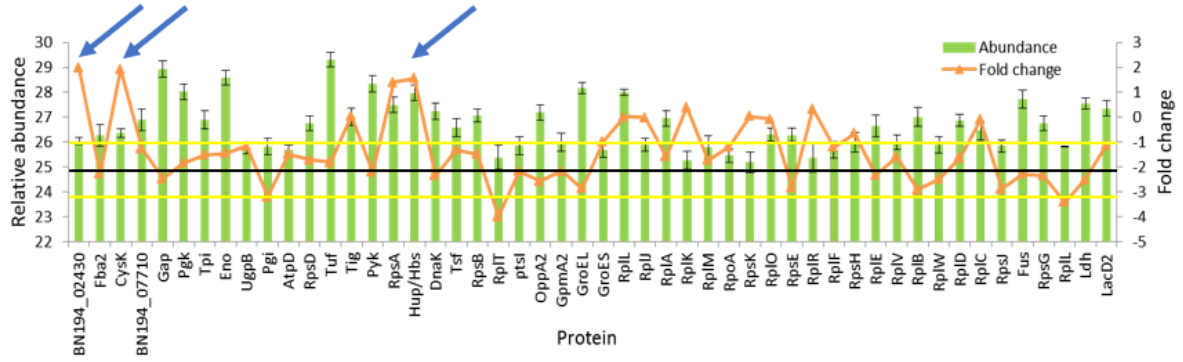


Figure 5. 2. Relative and differential abundances of selected proteins detected in the lithium chloride-sucrose (LS) fractions prepared from *L. casei* GCRL163 cells harvested at mid-exponential and stationary growth phases. LFQ values are $\log_2$-transformed and fold change is the difference between $\log_2$ LFQ for proteins detected in LS extracts at stationary when compared with mid-exponential phase. The error bars indicate standard deviations of triplicate samples of proteins detected at mid-exponential phase. The black line indicates the average change in abundance calculated across all of the proteins common to both growth phases and differences in the abundances were considered significant at $\log_2$-fold threshold of ≥ 1, $p < 0.05$, FDR 5% (yellow lines). Examples of proteins considered at significantly higher in stationary phase are marked (blue arrow).


LiCl-sucrose extracts
ML ST
-6.9 0 6.9
Trypsin Shaving extracts
ML ST
-5.7 0 5.7
ECF extracts
ML ST
-5.5 0 5.5

Figure 5. 3. Differential expression of proteins in different protein fractions obtained from *L. casei* GCRL163 cell surface at different growth phases. The heatmaps depict change in mean log_2-transformed LFQ intensity values from three technical replicates of cultures at mid-exponential (ML) and stationary (ST) growth phases in different protein fractions. The proteins were arranged based on their functional annotations. LFQ values were considered significant at log_2-fold threshold of ≥ 1 (upregulation) or ≤ -1 (downregulation), ($p<0.05$, FDR<5%) and proteins identified with minimum of two unique peptides at FDR<1% were represented.

Table 5. 1. Proteins expressed in high abundance in the *L. casei* GCRL163 cell surface fractions specific to growth phases. Highly abundant proteins in the lithium chloride-sucrose (LS), trypsin shaving (TS) and extracellular culture fluid (ECF) fractions specific to mid-exponential (ML) and stationary (ST) growth phases are represented. Proteins with mean log_2-transformed LFQ intensities ≥23 were considered significant (p<0.05, FDR<5%).

Protein ID	Gene locus	Protein name	Protein symbol	Predicted location*	Fraction/ growth phase where found
K0N3V9	BN194_11560	Glucosamine-fructose-6-phosphate aminotransferase	GlmS	CTP	TS, ST
K0MWS0	BN194_21340	dTDP-glucose 4,6-dehydratase	RmlB	CTP	LS, ML
K0N4K5	BN194_13650	Cell-shape determining protein Mbl	Mbl	CTP	LS, ML
K0N2F5	BN194_05410	Aminopeptidase N	PepN	CTP	LS, ML
K0N779	BN194_11580	Oligoendopeptidase F	PepF	CTP	LS, ML
K0MW64	BN194_18340	Glutamine synthetase	GlnA	CTP	TS, ST
K0NAC2	BN194_24680	Aminopeptidase C	PepC2	CTP	TS, ST
K0N634	BN194_19300	Phosphoribosylaminoimidazole carboxamide formyltransferase/inosine-monophosphate cyclohydrolase	PurH	CTP	TS, ST
K0N5S8	BN194_19360	Phosphoribosylaminoimidazole-succinocarboxamide synthase	PurC	CTP	TS, ST
K0NAP0	BN194_15970	DNA topoisomerase 1	TopA	CTP	LS, ML
K0N8A7	BN194_26500	RNA binding protein, S1_like superfamily	YabR	CTP	TS, ML
K0N8Z3	BN194_07570	Alpha-crystallin domain heat shock protein	Hsp18	CWP	LS, ML
K0MVC6	BN194_15340	ATP-dependent Clp protease ATP-binding subunit ClpX	ClpX	CMP	LS, ML
K0N7I8	BN194_26460	ATP-dependent zinc metalloprotease FtsH	FtsH	CMP	LS, ML
K0MSP8	BN194_04740	NADH peroxidase	Npr	CTP	TS, ST
K0NA95	BN194_23980	YbaB/EbfC DNA-binding family protein	BN194_23980	CTP	LS, ML
K0N8I3	BN194_27250	DUF1002 superfamily protein	YpuA	CWP	TS, ML
K0MT60	BN194_06940	PTS system beta-glucoside-specific transporter subunit IIBCA	BglP	IMP	LS, ML
K0N4S1	BN194_15410	PTS (fructose family) subunit IIABC	FruA3	CMP	LS, ML
K0MT56	BN194_06890	Multidrug ABC-type transporter permease/ATP-binding components	YfiB	IMP	LS, ML
K0N2V5	BN194_07700	ABC-type transporter, substrate-binding protein	BN194_07700	CWP	TS, ML
K0N5I3	BN194_17910	Oligopeptide transport ATP-binding protein OppF	OppF	CMP	LS, ML
K0N6C6	BN194_20600	Oligopeptide ABC-type transporter ATP-binding protein	OppD2	CMP	LS, ML;TS, ST
K0N307	BN194_08400	Acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase	AdhE	CTP	LS, ML
K0N7I6	BN194_12580	Glucose-6-phosphate isomerase	Pgi	CTP	TS, ST
K0NAN3	BN194_26330	DNA-directed RNA polymerase subunit beta'	RpoC	CTP	LS, ML
K0N3Z3	BN194_11600	DNA-binding regulatory protein, YebC/PmpR family	BN194_11600	CTP	TS, ML
K0MUM1	BN194_12190	TetR family transcriptional regulator	BN194_12190	CMP	LS, ML
K0MT67	BN194_06990	Cold shock protein 1	Csp	CTP	TS, ML
K0N6T2	BN194_00120	30S ribosomal protein S18	RpsR	CMP	LS, ML
K0NB12	BN194_17770	50S ribosomal protein L19	RplS	CTP	TS, ST
K0N697	BN194_20200	50S ribosomal protein L33	RpmG	CTP	LS, ML
K0MXR0	BN194_25840	30S ribosomal protein S9	RpsI	CWP	LS, ST
K0NCJ0	BN194_25970	30S ribosomal protein S11	RpsK	CMP	TS, ML
K0NAK7	BN194_26030	50S ribosomal protein L15	RplO	CTP	TS, ST
K0MXS9	BN194_26090	30S ribosomal protein S14 type Z	RpsZ	CTP	LS, ML
K0N869	BN194_26200	50S ribosomal protein L23	RplW	CTP	TS, ST
K0N6K2	BN194_08480	Alanine-tRNA ligase	AlaS	CTP	LS, ML
K0NA01	BN194_11970	Aspartyl/glutamyl-tRNA amidotransferase subunit B	GatB	CTP	LS, ML
K0N4J6	BN194_14510	Valine-tRNA ligase	ValS	CTP	LS, ML
K0N4L9	BN194_14810	Isoleucine-tRNA ligase	IleS	CTP	LS, ML
K0N5D3	BN194_16950	Glycine-tRNA ligase subunit beta	GlyS	CTP	LS, ML
K0N9N4	BN194_20080	Serine-tRNA ligase	SerS	CTP	LS, ML

*Protein subcellular localization was assigned according to subcellular localization prediction tools: integral membrane proteins (IMP) were predicted using TMHMM server 2.0 algorithm (Krogh *et al*. 2001). Cytoplasmic proteins (CTP), cell wall-associated/extracellular proteins (CWP) and cell membrane-anchored proteins (CMP) were predicted by cell-Ploc 2.0 (Chou & Shen 2010) and PSORTb (Nancy *et al*. 2010).

CHAPTER 6

QUANTITATIVE PROTEOMICS OF *LACTOBACILLUS CASEI* GCRL163 REVEALS KEY PROTEINS INVOLVED IN THE REGULATION OF PROLONGED HEAT STRESS RESPONSE

6.1 Abstract

The regulation of prolonged heat stress response (PHSR) is important for adaptive responses that adjust cellular and molecular functions to maintain energy production and structural integrity of macromolecules and enzymes during prolonged heat stress. To investigate the induction of the regulatory proteins involved in PHSR in *L. casei*, a forensic analysis was performed on LFQ proteomic datasets of the CFEs, cell surface-associated (TS and LS extracts) and ECF protein fractions of *L. casei* GCRL163, cultured anaerobically at pH 6.5 in bioreactors to mid-exponential phase at 30 to 45°C. The analysis revealed that HrcA played a central regulatory role in PHSR while CtsR was not detected in any fraction. The RNA polymerase subunits α, β and δ were detected in CFEs and at the cell surface as highly up-regulated after culture at supra-optimal temperatures and sigma factor σ^A (σ^{70}) was moderately up-regulated in the CFEs, while sub-units β′, ω and presumptive ε were down-regulated at 45°C, suggesting modulation of RNA polymerase was involved in PHSR . YycF ortholog inhibitors, considered as attractive bactericidal agents against pathogenic bacteria, could impact the ability of *L. casei* GCRL163 to cope with elevated growth temperature, as response regulator YycF_2 was induced at 45°C. Orthologs of proteins under the regulation of σ^B in *Bacillus subtilis* were up-regulated at 45°C, including Asp23-domain proteins Asp23_2, YqhY and uncharacterized protein BN194_17970. Several proteins associated with transcription, translation and post-translational modification were differentially modulated by thermal stress. This study shows the importance of proteomics in investigating stress

response regulation, which can complement the current transcriptomic-based knowledge in lactobacilli, particularly in identifying novel condition-specific regulators.

Keywords: Regulators, *Lactobacillus casei*, proteomics, prolonged heat stress response, probiotics.

6.2 Introduction

Lactic acid bacteria (LAB), such as members of the *Lactobacillus casei* group, are used as probiotics or starters in fermented food products, and are often exposed to several stressful conditions, including cold, heat, osmolarity extremes, acidity, alkalinity and nutrient fluctuations, during product manufacture and gastrointestinal passage (Bucka-Kolendo & Sokołowska 2017; Papadimitriou *et al*. 2016). Temperature fluctuations can involve prolonged exposure of *Lactobacillus* species to mild non-lethal heat stress conditions, with the induction of prolonged heat stress response (Papadimitriou *et al*. 2016), or a rapid transient upshift in the growth temperature, which elicits the heat shock response (HSR) (De Angelis *et al*. 2004). Bacterial cells have developed a cascade of genetic mechanisms which assist them to survive under stressful conditions (Mills *et al*. 2011a). These adaptive mechanisms involve differential gene expression that elicits the induction of specific sets of proteins (Corcoran *et al*. 2008). For instance, heat shock proteins (HSPs), including the molecular chaperones of the GroESL and DnaKJ complexes and related HSPs, are induced in response to heat shock in *L. casei, L. helveticus* and *L. acidophilus* (Broadbent *et al*. 1997). These chaperones are important for proper folding of cellular proteins in stressed and non-stressed states (Sugimoto & Sonomoto 2008). Irreversibly misfolded and denatured proteins are degraded by Clp ATP-dependent proteases (Savijoki *et al*. 2006). The heat shock response (HSR) involves a complex network of events which require coordinated regulation

for prompt response and eventual adaptation to harsh conditions (De Angelis *et al*.2004). The regulation of HSR has been extensively studied in model organisms such as Gram-negative *E. coli* (Bukau 1993) and Gram-positive *B. subtilis* at the transcriptomic level (Schumann *et al*. 2002). The *in silico* genomic analysis of HSR regulation in *L. casei* and *L. rhamnosus* has also been reported (Rossi *et al*. 2016). However, little is known about the regulation of PHSR in *Lactobacillus* species at the proteomic level.

From the transcriptional point of view, HSR regulation involves two major mechanisms that regulate the expression of heat shock genes and these include the transcriptional repressors or activators and alternative sigma (σ) factors (Schumann 2016). The transcriptional repressors include the HrcA and CtsR, which negatively regulate the heat shock genes including the *groESL* and *dnaKJ* operons and the genes encoding some Clp protease complexes (Schumann 2016). In *B. subtilis*, the class I heat shock genes (*groESL* and *dnaKJ* operons) are regulated by the transcriptional repressor HrcA through binding to the Controlling Inverted Repeat of Chaperone Expression (CIRCE) operator (Schulz & Schumann 1996). Promoters with inverted repeats homologous to CIRCE have also been identified in several *Lactobacillus* species (De Angelis *et al*. 2004), including upstream of the *dnaJ* gene in the *hrcA-grpE-dnaK* operon of *L. sakei* LTH681 (Schmidt *et al*. 1999), the *hsp16* gene of *L. acidophilus* (Capozzi *et al*. 2011) and the *clpL* gene of *L. rhamnosus* (Suokko *et al*. 2005). The CtsR (class III stress repressor) regulon, comprised of the *clp* operons including *clpC, clpP* and *clpE*, recognizes a tandemly repeated heptad operator sequence thereby serving as class III heat shock gene repressor in *B. subtilis* (Derré *et al*. 1999). The stress-responsive CtsR regulon has been studied in other Gram-positive bacteria such as *L. plantarum* (Fiocco *et al*. 2010) and *Oenococcus oeni* (Grandvalet *et al*. 2005). The class IV heat shock genes, such as *htpG* in *B. subtilis*, are under the control of other unknown

regulatory systems (Versteeg *et al*. 2003). In addition to HrcA and CtsR, two other repressors have been identified in *Streptomyces albus,* including the heat shock protein repressor (HspR) and repressor of *hsp18* (RheA) expression. The HspR binds to HspR-associated inverted repeat (HAIR) and represses the *dnaKJ* operon while RheA binds to an inverted-repeat sequence to repress the expression of *hsp18* (Servant *et al*. 2000). Furthermore, all bacteria code for at least one housekeeping σ factor, which regulates a large number of genes necessary for bacterial growth, and the nature of the housekeeping σ factor may vary among species. However, bacterial genomes normally contain several alternative σ factors, which are induced under particular stress conditions (Kazmierczak *et al*. 2005). The stress-response alternative sigma factor σ^B has been characterized in other Gram-positive bacteria including *Listeria monocytogenes* (Chaturongakul & Boor 2006) and *Staphylococcus aureus* (Cebrián *et al*. 2009). Notably, alternative σ^{32} and σ^{24} have been identified as positive regulators of the HSR in *E.coli* (Schumann 2016) and in an *in silico* study of *L. casei* (Rossi *et al*. 2016). Several genes predicted as members of the σ^{32} regulons are involved in cellular adaptive mechanisms such as cell structure maintenance, translation and transcription systems necessary for nascent peptide maturation, transporter and carbohydrate metabolism (Kormelink *et al*. 2012; Rossi *et al*. 2016).

The proteomic data in this study revealed the expression of regulatory proteins involved in the positive and negative regulation of PHSR in *L. casei* GCRL163. Several proteins encoded by regulons which are controlled by the regulators are identified while some key regulators found in the genome of *L. casei* GCRL163 were not detected. The findings indicate that several regulatory proteins are involved in cellular processes associated with PHSR that facilitates the adaptation of *L. casei* GCRL163 to prolonged heat stress.

6.3 Results

6.3.1 Regulators of prolonged heat stress response are modulated in *L. casei* GCRL163 at elevated temperatures

Several proteins involved in regulatory systems were detected in this study and were differentially expressed at 35°C, 40°C and 45°C relative to cells cultured at 30°C (Table 6. 1). The cell membrane-anchored, heat-inducible transcription repressor HrcA detected in the CFE and LS protein fractions showed moderately increased abundance at 35°C and no differential abundance at higher temperatures in the CFEs.

The proteins encoded by the class I stress response genes detected included GroES and GroEL encoded by the genes that constitute the *groESL* operon (Walker *et al*.1999) and showed increased expression at the elevated growth temperature of 45°C in CFEs, with similar upregulation detected for GroEL in the TS fractions (Table 6. 1). GroEL was highly induced at 45°C with relatively small fold-changes at 35°C and 40°C in the CFE and TS while overexpressed at all the growth temperatures in the LS fractions. GroES showed abundant change at 45°C in the CFE and LS fractions. Other molecular chaperones, associated with regulation by the transcription repressor HrcA, were the heat shock proteins of the Hsp70 complex encoded in the *dnaK* operon (Schmidt *et al*.1999) including GrpE, DnaK (both increased at 45°C) and DnaJ, which was increasingly up-regulated at all the growth temperatures above 30°C in the CFEs. In the TS fractions, DnaJ was also over-expressed at all the growth temperatures while GrpE was moderately increased at 35°C and 40°C and repressed at 45°C. The transcriptional repressor CtsR, which is often responsible for the negative regulation of the Clp system and other molecular chaperones in Gram-positive bacteria (Derré *et al*. 1999), was not detected in the CFE, ECF and surface-

associated protein fractions at any growth temperatures under consideration (Table 6. 1) or stage of growth (data not shown), although the gene is in the genome of *L. casei* GCRL163 (Nahar *et al*. 2017). This could be due to undetectable LFQ intensity, lack of expression or degradation by ClpP proteases (Frees *et al*. 2007). The majority of the Clp proteases were over-expressed at the elevated temperature of 45°C in *L. casei* GCRL163 in the CFEs and they include ClpP_2, ClpA/B, ClpB and ClpE (Table 6. 1). ClpC was found to be up-regulated at the growth temperatures of 40°C and 45°C while ClpX and ClpB_2 were not differentially modulated by growth at elevated temperatures. Furthermore, ClpB, ClpC, ClpE and ClpP_2 were observed as over-expressed in the TS fractions at 40°C and 45°C while ClpB was increased at 45°C in the LS fractions.

Several transcriptional proteins were modulated in *L. casei* GCRL163 under prolonged heat stress. Cytoplasmic protein DNA-dependent RNA polymerase (RNAP) sigma factor σ^A (σ^{70}, RpoD) demonstrated slightly elevated expression at all temperatures above 30°C in CFE fractions (Table 6. 1). RNAP subunit α (RpoA), which forms part of the core subunits of RNAP, and subunit δ (RpoE), showed $\log_2$1.6- and 1.3-fold change increase respectively at the growth adaptation of 45°C in CFEs. RpoA was also increased at 40°C and 45°C ($\log_2$ 3.04) while RpoE increased ($\log_2$ 2.1) at 45°C in the TS fraction. Other core RNAP subunits β (RpoB) and β' (RpoC) were not differentially modulated in the CFEs, although RpoB was up-regulated at 45°C in the TS fraction. However, RNAP ω subunit (RpoZ), the smallest core RNAP subunit, was repressed at 45°C in the CFEs and not detected in the TS fraction.

Alternative σ factors are recruited and employed in the transcription initiation of specific subset of genes encoding various proteins in bacteria (Mooney *et al*. 2005). Although

alternative σ factors were not detected in the study, protein orthologs which are under the regulatory control of σ^B in *B. subtilis* and *S. aureus* (Müller *et al*. 2014) were detected (Table 6. 2). This included three proteins containing the Asp23 domain: Asp23_2 (alkaline-shock protein), YqhY and uncharacterized protein BN194_17970. Others included YhjA, Hpf and BN194_28350 (DgaF). Asp23_2 was only detected at 45°C, at moderately high abundance, while YqhY was over-expressed at 40°C in the CFEs. Cell wall-anchored protein BN194_28350 demonstrated a similar pattern of upregulation at 40°C and 45°C in the CFE and LS fractions. In the TS fraction, YhjA was highly up-regulated at 45°C. Although *Lactobacillus* species are considered to lack the equivalent of the *B. subtilis* σ^{B2}, which is involved with transition into stationary phase and other stress responses, these observations indicate that the targets of this alternative sigma factor in *B. subtilis* and other Gram-positive species are differentially expressed following growth at supra-optimal temperature.

6.3.2 Proteins associated with sensing environmental stimuli are modulated by prolonged heat stress

Sensing environmental stimuli and prompt reprogramming of specific gene transcription, through the activation of two-component signal-transducing systems, are important components of responding to thermal stress (Marles-Wright & Lewis 2007). In the current analysis, the two-component system response proteins detected included the cytoplasmic proteins YycF_2, YclJ, BceR_2, ArlR, VanR and PhoP (Table 6. 3). These proteins possess OmpR/PhoB-type domains which constitute the largest subfamily of response regulators, which bind to DNA through the C-terminal effector domain (Kenney 2002). The response regulators YycF_2 and BceR_2 showed approximately $\log_2$1.0-fold change increase at 45°C and 40°C respectively while YclJ and PhoP were repressed at 45°C in the CFE. One of the universal stress (UspA superfamily) paralogs (BN194_13870)

detected was over-expressed at 40°C and 45°C and integral membrane protein BN194_08710, with divergent methyl-accepting chemotaxis-like domain, at 40°C in the CFEs. We identified sensor protein CesK/VanS, possessing histidine kinase domain, in the LS fractions, although abundance was not impacted by prolonged heat stress. Also, our proteomic data revealed cell wall stress sensing response regulator LiaR involved in cell wall stress signal transduction. Prolonged heat stress at 40°C and 45°C induced repressed expression of LiaR with CesR/VanR showing no differential expression during prolonged heat stress in LS and CFE fractions.

6.3.3. Proteins associated with transcriptional regulation

The majority of the proteins associated with transcriptional regulation in *L. casei* GCRL163 belong to different families of regulatory proteins and demonstrated differential expression in one or more proteins fractions (CFEs, LS and TS), depending on the subcellular locations of the proteins. Several of the regulators detected were demonstrated to show moderate or no differential expression (Table 6. 3). However, there were some regulators that showed high differential expression during prolonged heat stress. Notably, HTH_XRE transcriptional regulator BN194_10050 and ArsR family transcriptional regulator BN194_22710 were highly up-regulated at elevated growth temperature in the CFEs while YodB was over-expressed in the LS fraction. ArsR could be involved in the regulation of metal ion efflux such as arsenic ions (Wu & Rosen 1991). Others including cell wall-associated transcriptional regulator LytR-CspA-Psr superfamily LytR_3 and LytR-CspA family transcriptional regulator LytR_4 were highly down-regulated in the LS at 45°C. Moreover, MerR family transcriptional regulator BN194_25900, CBS domain-containing GntR family transcriptional regulator YtoI and HTH_XRE family transcriptional regulator BN194_13860 were highly repressed in LS fraction while DNA-binding regulatory protein

YebC/PmpR family BN194_11600 was repressed in TS fraction at 45°C. MerR-type transcriptional regulators are bacterial transcription activators which are induced by stress conditions including heavy metal (e.g. mercury), antibiotics and oxidative stress while being increasingly recognized as regulating broader metabolic processes (Brown *et al*. 2003; Schumacher *et al*. 2018). Other proteins associated with transcription were detected and included termination factor NusB which was repressed at 40°C and 45°C while transcription elongation factor GreA was repressed only at 45°C in the CFEs (Table 6. 3). The transcription elongation protein NusA was less abundant while NusG was over-expressed at 45°C in the TS fraction. Cold shock proteins Csp and CspLA had repressed expression at 40°C and 45°C prolonged heat stress

6.3.4. Proteins associated with DNA replication and repair

Proteins related to DNA replication initiation, elongation and topological change were impacted by prolonged heat stress mostly in the CFEs (Table 6. 3). DnaC and Ssb, associated with DNA replication initiation, were moderately up-regulated during prolonged heat stress while DnaA and Obg showed repressed expression mostly at 45°C in the CFEs. LigA and DnaX, involved in DNA replication elongation, were highly down-regulated and the expression of DnaN was highly enhanced at 45°C in the CFEs. Some proteins, including GyrA and ParE involved in DNA topological change, demonstrated repressed expression while others such as Hup and TopA were up-regulated during prolonged heat stress in different fractions.

Furthermore, most of the proteins involved in the regulation of PHSR and associated with DNA repair and recombination were repressed during prolonged heat stress (Table 6. 3). DNA polymerase I, PolA was repressed at the growth temperature of 40°C and 45°C

respectively. Also repressed expression of PcrA and MutS at 40°C and 45°C was observed. Recombination DNA repair protein RecR was only repressed at 40°C while recombination and DNA strand exchange inhibitor MutS2 showed repressed expression at 45°C in the CFEs. However, $\log_2$1.9 fold-change increase of UvrB was observed at 40°C.

LexA was suppressed beyond detection at 45°C in the CFE. LexA is involved in the repression of several proteins associated with the response to DNA damage, including RecA and LexA, and several genes involved in the SOS response (Little *et al*. 1981). Although RecA was over-expressed at 40°C and 45°C in the TS fraction, the protein was less abundant at 45°C in the CFE. In the presence of single-stranded DNA, RecA and LexA interact to cause autolytic cleavage of LexA (which is an ArsR-like repressor with peptidase activity) leading to repression of the SOS regulon. In *B. subtilis*, the *yneA* operon is a small SOS operon under the control of LexA which is responsible for cell division suppression during the SOS response (Kawai *et al*. 2003). We detected an uncharacterized BN194_17750 only in the LS fraction (dataset identifier PXD009591), where it was up-regulated ($\log_2$ 2.3-fold): it contains a DUF896 (UPF0291, pfam 05979) domain also found in Yne proteins (UniProt). The genomic location was between two proteins that form an operon, BN17740 (UPF0154, transmembrane, up-regulated at 40°C and 45°C in the CFEs) and BN194_17760 (uncharacterized, not detected). The role of these proteins is experimentally unknown and their regulation by LexA unexplored, although this would be an interesting line of research in the future.

6.3.5 Modulation of proteins involved in translation under prolonged heat stress

Several proteins involved in translational processes in *L. casei* GCRL163 demonstrated varied pattern of modulation in the protein fractions at different growth

temperatures (Table 6. 3). Most of these proteins are associated with translation initiation, elongation, release and attenuation. Expression of the proteins linked to the translation initiation such as translation initiation factors InfA, InfB and InfC, were not differentially modulated during prolonged heat stress in the CFEs. However, in the TS fraction, InfA was over-expressed at 40°C and 45°C while InfC was up-regulated at 45°C in both LS and TS fractions. Conversely, InfB was less abundant at 40°C and 45°C in the TS and at 45°C in the LS fraction. Most of the translation elongation-associated proteins were up-regulated at the elevated temperature of 45°C in the CFE, including elongation factors Tuf, Fus and Tsf. Moreover, Tuf and Tsf were over-expressed at 40°C and 45°C and Fus increased at 45°C in the TS protein fraction. Elongation factor Efp was however repressed at 45°C in the CFE. The detected proteins involved in translation release include the peptide chain release factor 1 (PrfA), 2 (PrfB) and 3 (PrfC). These proteins showed abundant expression during prolonged heat stress with PrfB demonstrating $\log_2$ 2.3-fold change increase at 45°C in the CFE. EngD, with putative role in translation attenuation and peptide release, was over-expressed in the LS fraction while SmpB, involved in rescuing stalled ribosomes, was up-regulated in the CFE and LS fractions at 45°C.

The majority of ribosomal proteins detected in *L. casei* GCRL163 were not differentially modulated during prolonged heat stress in the CFE (Table 6. 3). However, several of them were over-expressed mostly at the elevated growth temperatures in the TS and only few in the LS fractions. Notably, in the CFE fraction, RpsA and RpsR were up-regulated while RplF and RplI were repressed at 45°C. In the TS fraction, RplK, RpsX and RplW were over-expressed at all the growth temperatures, RplR at 35°C, RplJ, RpsK, RplO, and RplV at 40°C and 45°C, and RpsR, RplT, RplF, RplE, RpsS, RplB, and RplD at 45°C. However, RplV was less abundant at 35°C, RplA, RpsJ and RpsG were repressed at 40°C,

RpsC at 40°C and 45°C, and RpsD at 45°C in the TS fraction. Ribosomal proteins over-expressed at 45°C in the LS fraction included RplK, RpsX and RplW.

Furthermore, prolonged heat stress led to differential expression of proteins associated with tRNA/ribosome assembly and processing in *L. casei* GCRL163. YabR and YpsC were repressed at 40°C and 45°C in the CFEs. Similarly, YabO, RsmA, Rnc, Rnr and MnmA, were repressed at 45°C in CFEs. However, other proteins showed enhanced expression at 35°C (RsmF), 40°C (Cca, RnpA and RbgA) and 45°C (YtpR) in the CFEs. Moreover, RnpA, YabO and MnmE were up-regulated at 45°C in the LS while YabR was repressed at 40°C and 45°C in the TS fractions.

Most of the proteins associated with tRNA charging detected in this study were repressed by prolonged heat stress in the CFEs. Some of the examples include PheT, PheS, GltX and AlaS, which were repressed at the elevated temperature of 45°C (Table 6. 3). The protein AlaS was up-regulated however at 45°C in the TS fraction. We also detected modulation of proteins involved in RNA degradation and include the Rny, involved in mRNA decay (riboswitch turnover), RnjA and RnjB. The protein Rny was over-expressed at 40°C, RnjB at 40°C and 45°C, and RnjA at 45°C in the CFEs. However, RnjA was less abundant at 40°C in the CFE while RnjB was enhanced at 45°C in the TS fraction.

6.3.6 Proteins involved in post-translational modification are modulated by prolonged heat stress

Proteins associated with post-translational modification detected were mostly over-expressed in the LS fractions at elevated growth temperature of 45°C. Notably, cell wall-associated protein thioredoxin family proteins TrxA_2 and YtpP, and cell wall-associated

protein tyrosine and serine phosphatase BN194_29310 involved in protein phosphorylation, were up-regulated in the LS fractions. Moreover, thioredoxin reductase TrxB was enhanced at 45°C and SmpB at all the growth temperatures in the CFE. However, cell wall-associated protein Ptp3 was repressed at 40°C and 45°C, TrxA_2 and YtpP at 45°C in the CFE. The Ptp3 was also less abundant at 45°C in the LS protein fractions.

6.3.7 Other proteins associated with the regulators of prolonged heat stress response are modulated by the heat stress

Several other proteins identified in the current analysis are linked to several regulatory functions. Proteins associated with cytokinesis including, Noc (increased at 35°C) and ParB were detected (Table 6. 3). Z-ring protein bundle promoter protein BN194_0854, septation ring formation regulator EzrA were up-regulated at 40°C and GpsB, involved in the regulation of PBP1 localization during cell cycle progression, at 40°C and 45°C in the CFEs. However, Smc was repressed at 40°C and 45°C in the CFE and EzrA at 45°C in the LS fractions. Other proteins with unknown functions including YaaA, Eep, CinA, and YitL were repressed at high growth temperatures in various fractions. YbaB/EbfC BN194_23980, CBS pair domain protein BN194_26610 and YeaO were however up-regulated at elevated growth temperature of 45°C in the fractions (Table 6. 3).

6.4 Discussion

The knowledge of the regulators involved in PHSR is still scant particularly at the proteomic level. *L. casei* cells are exposed to heat shock and persistent heat stress, to which they often respond by eliciting various adaptive mechanisms that involve complex modulation of protein expression (Papadimitriou *et al*. 2016). Furthermore, several of the regulatory proteins were not only detected in the CFEs but also in the LS, TS and ECF

fractions, suggesting their involvement in regulatory functions or response to regulatory changes at the cell surface. Changes in protein expression of some proteins were not obvious in the CFE fraction and could only be detected at the cell surface. The two-component signalling systems play a central role in sensing adverse environmental conditions through an integral membrane histidine protein kinase (Hpk) acting as a sensor, which is coupled to a cytoplasmic response regulator (RR) or an effector that mediates the eventual expression of genes encoding proteins associated with stress responses (West & Stock 2001). The interaction between the Hpk and RR is mediated through phosphorylation and dephosophorylation reactions (Stock *et al*. 2000). The two-component signalling systems are involved in the regulation of key physiological processes with differences existing in the regulatory networks under the control of homologous two-component signalling systems in different species of lactobacilli or those of other related bacteria (Alcantara *et al.* 2011). Although the histidine kinase domain-containing protein VanS was not impacted during prolonged heat stress, some of the OmpR/PhoB-type domain-containing proteins, including response regulators YycF_2, BceR_2 and one of the universal stress proteins detected, UspA, was enhanced and could be vital for transducing signals during thermal stress. YycF has been linked to the regulation of fatty acid biosynthesis in *S. pneumoniae* by binding to the regulatory region of the gene encoding FabT (a fatty acid biosynthesis repressor), depending on its state of phosphorylation, and inhibiting FabT transcription (Mohedano *et al*. 2016). YycF was reported to be highly conserved and specific to low G+C Gram-positive bacteria such as *B. subtilis* and *S. aureus*, playing key roles in cell wall metabolism and cell viability (Bisicchia *et al*. 2007; Dubrac *et al*. 2007). Consequently, novel antimicrobial agents targeting YycF were considered attractive and could have bactericidal effects on a wide range of pathogenic bacteria (Gotoh *et al*. 2010). Inactivation of YycF homolog-encoding gene *rr-p3* in *L. sakei* or its ortholog RR16 in *L. casei* did not produce any remarkable growth defect

despite its homolog YycFK/WalRK system was demonstrated to be essential for growth in bacilli and staphylococci (Alcantara *et al.* 2011; Morel-Deville *et al*. 1998). However, the lack of phenotypic effect observed under the experiemental conditions in *L. sakei* and *L. casei* does not exlude the fact that YycF may play essential role under particular growth conditions (Monedero *et al.* 2017). In the current study, YycF family protein was induced at 45°C in *L. casei* GCRL163, suggesting that an inhibitor of YycF could impact the ability of the strain to cope with growth at elevated temperature.

Our results indicated that expression of genes encoding proteins from a variety of functional classes was induced during prolonged heat stress (Chapter 3 and 4), where expression is likely important for protecting cells from the adverse effects of thermal stress. The expression of these genes is usually under the control of regulators. The *in silico* genomic analysis of thirteen probiotic strains of *L. casei* and *L. rhamnosus* revealed the identification of the HSR regulators, HrcA and CtsR transcriptional repressors, and alternative σ^{24} and σ^{32} homologs in all of the genomes but with $\sigma^{70/24}$ and a Lon protease present only in some genomes (Rossi *et al*. 2016). The transcriptional repressor HrcA appeared to play a central role in the regulation of PHSR in *L. casei* GCRL163. Although the expression of the transcriptional repressor HrcA was not differentially modulated by prolonged heat stress, proteins encoding the operons *dnaKJ* and *groESL* complex were up-regulated at the elevated growth temperatures, as occurs in the HSR of Gram-positive bacteria (Rossi *et al*. 2016; Schmidt *et al*. 1992). During growth at non-stressing temperatures, the chaperones interact with the HcrA protein so the repressor is maintained in an active conformation, which can bind to the CIRCE to block transcription (Schumann 2016). Under heat stress, the chaperones are titrated by misfolded and denatured proteins, so

the HrcA protein is no longer stabilized and the inactive repressor fails to bind to DNA, allowing the RNAP holoenzyme to elicit transcription (Schumann 2016).

The alternative σ factors are recruited by the core RNA polymerase complex to initiate transcription and activate various adaptive responses (Österberg *et al*. 2011), although RNA polymerase subunits can also participate in all stages of transcription initiation (Borukhov & Severinov 2002). In *E. coli*, σ^{32} is associated with the normal expression of heat shock genes and heat shock response (Grossman *et al*. 1987). The σ^{54} was reported to play a crucial role in regulating bacteria-host interactions by regulating the gene expression involved in the transport and biosynthesis of precursors of exopolysaccharides, lipopolysaccharides, lipoproteins, peptidoglycan and lipids (Francke et al. 2011). Rossi *et al*. (2016) predicted the existence of alternative factor σ^{32} and σ^{24} in *L. casei* and *L. rhamnosus*. We could not detect alternative σ factors (σ^{24}, σ^{32} and σ^{54}) and EpuA in all the protein fractions in the current study, despite their presence in the genome of the strain (Nahar *et al*. 2017). However, the detected uncharacterized protein UPF0122 BN194_17830 possesses sigma70_r3/4-like and YlxM-like domains which may be important in the regulation, especially in signal recognition particle pathway, as may other proteins with sigma70-like domains. Despite not detecting σ^{54} in this study, we identified a ribosome hibernation promoting factor Hpf with σ^{54} modulation domain, which was repressed at elevated temperatures. The cytoplasmic protein Hpf is involved in ribosome hibernation of 100S during stress conditions, including stationary growth phase in *E. coli* (Ueta *et al*. 2008) and ribosome dimerization in *B. subtilis* (Akanuma *et al*. 2016). Its lowered expression levels at elevated temperatures, failure to detect the integral membrane protein CelR (which positively controls σ^{54} to allow binding to promoters in response to stress) and no expression of σ^{54}, indicate minimal or no involvement of σ^{54} in PHSR in *L. casei* GCRL163. However, we did

observe differential expression of some of the core RNAP subunits: α (RpoA), β (RpoB) and β' (RpoC), as well as δ (RpoE) and ω (RpoZ), which were detected in CFE and surface protein extracts with noted upregulation of RpoA, RpoE and RpoB at the cell surface (TS fractions) and RpoA and RpoE in the CFEs. Moreover, the detected σ^A/RpoD was slightly more abundant in all CFEs at all temperatures >30°C, which suggests σ^A/RpoD may be involved in the regulation of PHSR, as a housekeeping σ factor. We failed to detect σ^{32} in any extracts, which also may indicate a lesser or no role in modulating gene expression during heat stress in this strain. In *E. coli*, σ^{32} is subject to proteolysis by enzymes, including Clp proteases and FstH, depending on its binding relationship with DnaJ and DnaK (Suzuki *et al*. 2012), which may also account for undetectable LFQ intensity in this study particularly in view of the upregulation of several proteases (Table 6. 1). These findings suggest that the RNA polymerase subunits also play central roles in various processes of transcription, including the initiation of transcription of stress-related operons during thermal stress in the absence of expressed alternative σ factors. The role of RpoE in RNAP function in *Lactobacillus* species, a subunit confined to firmicutes, has not been explored, although it was shown to be involved in rapid adaption, survival, growth phase transition and competitive fitness of *B. subtilis* cells while not being essential (de Saro *et al*. 1999; Rabatinová *et al*. 2013). We suggest here that the observed altered ratios of the core RNAP subunits in *L. casei* GCRL163, and particularly the small, accessory RpoE protein in the cell surface, play a role in transcriptional specificity, in line with accumulating evidence of the importance of the small RNAP subunits in regulation in Gram-positive species (Weiss & Shaw 2015). To the best of our knowledge, this is the first report of the differential detection of RNAP subunits, especially RpoE, in the cell surface fractions in lactobacilli.

Our proteomic analysis advocates that the regulation of the Clp system in *L. casei* GCRL163 during prolonged heat stress may be under the control of the transcriptional repressor HrcA. The absence of the transcriptional repressor CtsR in all the protein fractions and at all the growth temperatures in the current study may suggest the limited involvement of the transcriptional repressor CtsR in PHSR in *L. casei* GCRL163. A recent genomic report on the *L. casei* GCRL163 revealed the presence of the transcriptional repressor CtsR in the genome of the strain (Nahar *et al*. 2017). The CtsR was not differentially detected in *L. casei* BL23 under bile stress (Alcantara & Zuniga 2012). The CtsR regulates class III genes which do not possess CIRCE operator sequence and the induction by stress conditions is independent of sigma-B (Derré *et al*. 1999). Fiocco *et al*. (2010) reported that CtsR controls ClpP expression in *L. plantarum* in response to environmental stress conditions. In *B. subtilis*, CtsR repressor can be degraded by the ClpCP and ClpEP protease complexes such that the suppression of proteins such as ClpC, ClpE, ClpP complex under its control is inhibited (Krüger *et al*. 2001). This might explain the non-detection of the CtsR repressor, in consistent with the over-expression of the ATP-dependent proteases ClpP, ClpC and ClpE mostly at 40°C and 45°C in various fractions in the current analysis. Furthermore, the expression of only one transcriptional repressor in the regulation of stress response is not uncommon, although dual HSR regulation by HrcA and CtsR has been reported in *S. salivarius* (Chastanet & Msadek 2003) and *S. aureus* (Chastanet *et al*. 2003). In the transcriptional analysis of *O. oeni* IOB8413, only an ortholog of the *ctsR* gene could be identified as the key regulator of stress response gene expression with the genome of the bacteria containing no other transcriptional repressor genes such as *hrcA* or CIRCE operator sequences (Grandvalet *et al*. 2005). The dual regulatory mechanisms of the transcriptional repressors HrcA and CtsR demonstrate synergy rather than redundancy in the control of the stress response genes (Schumann 2016).

The proteomic analysis further reveals several other proteins that are targets of key regulators of PHSR in *L. casei* GCRL163. Many of these proteins are involved in network of metabolic and cellular processes necessary for the adaptation of the bacterial cells during the heat stress. Of significant importance are the transcriptional proteins. The LytR family protein could be responsible for the maintenance of cell wall structures through the regulation of autolysins in *Streptococcus mutans* (Chatfield *et al*. 2005). We identified cell wall-anchored proteins LytR_3 and LytR_4, which possess LytR_CpsA_Psr and LytR_CpsA domain respectively. Proteins possessing LytR_CpsA-Psr domain have been described as extra-cytoplasmic transcriptional attenuator, required for teichoic acid and capsular polysaccharide transport to the cell wall peptidoglycan in *B. subtilis* (Kawai *et al*. 2011). LSEI_0247 regulator, belonging to the LytR_Cps2A_Psr, is a cell-envelope-related transcriptional attenuator in *L. casei* required for gut colonization (Licandro-Seraut *et al*. 2014). Although the regulatory roles of the MarR family transcriptional regulators BN194_08230 and BN194_22670 are unknown, proteins possessing the domain have been associated with the negative regulation of antibiotic resistance and chemical stress response in *E. coli* (Alekshun & Levy 1999). They are also involved in the regulation of bile salt response in *L. plantarum* (Bron *et al*. 2004). Other transcriptional regulators identified include the GntR, a repressor of gluconate operon in *B. subtilis* (Haydon & Guest 1991), MerR, involved (amongst other roles) in metal response (e.g. mercury) and detoxification (Hobman *et al*. 2005) and ArsR, associated with arsenical and metal resistance regulation (Wu *et al*. 1991). Uncharacterized YebC/PmpR-like protein BN194_11600 was repressed by thermal stress, suggesting that the protein was not actively involved in prolonged heat stress, despite previously observed upregulation of proteins involved in proteolysis and peptide transport (Chapter 3 and 4). However, YebC was shown to bind to the promoter region of

prtL, *oppA1* and *optS* genes in *L*. *deLrueckii* subsp. *lactis* CRL 581, thereby demonstrating its involvement in regulating the proteolytic system of this species (Brown *et al*. 2017). Cellular proteolysis has been associated with nutrient processing, polypeptide quality control and control of other regulatory processes in lactobacilli (Savijoki *et al*. 2006).

Furthermore, catabolite control protein A (CcpA) was reported as the central transcriptional regulator in carbon catabolite repression (CCR) needed for the regulation of sugar utilization and metabolism in most Gram-positive bacteria (Iyer *et al*. 2005). Glycolytic precursors induce HPr phosphorylation at the serine residue at position 46 with the resultant P-Ser-HPr interacting with catabolite control protein A (CcpA) to prevent catabolism of other carbohydrates (Papadimitriou *et al*. 2016). Phosphocarrier protein HPr was abundantly expressed at all growth temperatures in the TS and at 45°C in the LS while repressed at 40°C and 45°C in the CFEs. The ability of bacterial cells to make metabolic decisions especially in the presence of a wide variety of carbon and energy sources depends on the regulatory activities of CCR which represses the expression of genes specific for the utilization of non-preferred sugars until needed (Warner & Lolkema 2003). Regulation of CcpA was observed in the TS fractions with moderate inhibition at 35°C and 45°C and no differential modulation in the CFE and LS fractions. Although CcpA has not been previously reported to be detected in the cell surface of *L. casei*, detection in the cell wall fractions of *S. pneumoniae* has been documented (Iyer *et al*. 2005). To the best pf our knowledge, this is the first report of CcpA in the cell surface fractions in *L. casei*. Phosphocarrier protein HPr was abundantly expressed at all growth temperatures in the TS and at 45°C in the LS while repressed at 40°C and 45°C in the CFEs. In a previous study, we observed an upregulation of proteins involved in the uptake of various sugars other than glucose in the phosphotransferase systems while glycolytic enzymes and other proteins involved in scavenging alternative carbon sources

from other metabolic processes were over-expressed at the elevated growth temperature of 45°C in the mid-exponential phase *L. casei* GCRL163, despite culturing in a glucose-rich MRS broth medium (Chapter 3). This observation is consistent with the inhibition or no differential abundance of CcpA observed in the current analysis and suggests that the heat-adapted cells are relieved from catabolite repression as the cells require additional carbon sources to cope with the high energy-requiring processes needed for survival at elevated high temperature.

In this study, we have demonstrated the protein expression of the identified key regulators of PHSR and other proteins controlled by the regulatory proteins, which are involved in various metabolic and cellular processes during PHSR in *L. casei* GCRL163. Several of these proteins were repressed at the elevated growth temperature, including proteins involved in the tRNA aminoacyl synthesis (AlaS, PheS, PheT and GltX were repressed at 45°C in the CFEs while AlaS was up-regulated at 45°C in the TS) and tRNA/RNA assembly and processing (Rnr, MnmA, YpsC, YabR, YabO and RsmA repressed mostly at 45°C in the CFEs with YabR also repressed at 45°C in the TS while RnpA and YabO were up-regulated in the LS fractions at 45°C). Proteins associated with RNA degradation, including RnjA in the CFEs and RnjB in the CFE and TS fractions at 45°C with Rny at 40°C, were up-regulated. Several ribosomal proteins were enhanced in the TS surface protein fractions, although most were not differentially regulated in the CFEs with the majority of proteins associated with stages of translation processes showing overexpression in various fractions during prolonged heat stress. Proteins involved in post-translational modification were also impacted by the thermal stress (TrxA_2, YtpP, CobB and BN194_29310 in the LS and TrxB and CobB in the CFEs were up-regulated while TrxA_2, YtpP, Ptp3 were down-regulated in the CFEs at 45°C). SsrA-binding protein SmpB is a

RNA-binding protein that forms important component of the SsrA quality-control system and the protein is involved in stalled ribosome rescue during trans-translation in *E. coli* (Nonin-Lecomte *et al*. 2009). This protein is highly up-regulated especially at 45°C in the CFEs and LS and could be involved in the rescue of stalled ribosomes caused by thermal stress. We further detected repression of tyrosine-protein phosphatase Ptp3 at elevated growth temperature. Tyrosine-protein phosphatase is associated with the regulation of the biosynthesis of bacterial capsular and extracellular polysaccharides (Standish & Morona 2014). Prolonged heat stress repressed uncharacterized protein YitL (BN194_15600) is a conserved virulence factor B-like protein. The protein YitL regulates the expression of exoprotein genes in *S. aureus* (Matsumoto *et al*. 2007). Furthermore, Müller *et al*. (2014) reported that the transcription of alkaline shock protein Asp23 was regulated by alternative σ^B in *S. aureus* during alkaline shock. This protein is anchored by a small integral membrane anchor protein AmaP to the cytoplasmic membrane of *S. aureus* cells during the stress (Müller *et al*. 2014). Prolonged heat stress led to enhanced expression of the Asp23 domain-containing proteins YqhY, BN194_17970 and Asp23_2 at 40°C and 45°C growth temperatures, suggesting their involvement in prolonged heat stress response in *L. casei* GCRL163. However, only Asp23_2 was located near a presumptive AmaP homolog (annotated in *L. casei* GCRL163 genome (Nahar *et al*. 2017) with a locus layout similar to reported in *S. aureus* (Kuroda *et al*. 2001). The unique detection of Asp23_2 may also suggest cell surface structural changes, given the documented role of this class of stress protein in cell wall stress in *Staphylococcus aureus* (Müller *et al*. 2014) and the recent implication of this protein in cell surface changes in gentamycin resistant *L. casei* (Zhang *et al*. 2018). Several other proteins with unknown regulatory roles in PHSR were identified and many proteins with known regulatory roles as reported in model organisms such as *E. coli* or

B. subtilis would require further genomic analysis and experimental evidence to establish their mechanisms in *L. casei*.

In order to gain further in-depth insight into how the metabolic pathways of *L. casei* GCRL163 are impacted by the prolonged heat stress, expression of cytoplasmic and surface proteins at mid-exponential growth phase was investigated as described in Chapter 7.

6.5 Tables

Table 6. 1. Regulatory proteins of prolonged heat stress response in *L. casei* GCRL163 and their differential modulation under different growth temperatures.

Gene locus	Protein name	Protein symbol	35°C[a]	40°C	45°C	Function	Predicted localization[b]
			Log2 Ratio				
Regulators							
BN194_17480	Heat-inducible transcription repressor HrcA	HrcA	**0.99**	0.39	**0.51**	Regulates class I stress response genes	CMP
BN194_26360	Transcriptional repressor CtsR	CtsR	**ND**	ND	**ND**	Regulates class III stress response genes	CTP
Chaperones and protein folding							
BN194_07570	Alpha-crystallin domain heat shock protein[c]	Hsp18	**-5.32**	-5.19	**-5.24**	Protein disaggregation (during rapid growth)	CWP
BN194_08960	Peptidyl-prolyl cis-trans isomerase	PpiB	**-0.02**	-1.06	**-0.48**	Protein folding	CTP
BN194_17450	Chaperone protein DnaJ	DnaJ	**1.74** **2.30**	2.06 2.06	**3.32** **2.40**[d]	Nascent protein folding	CTP
BN194_17460	Chaperone protein DnaK	DnaK	**0.63**	0.48	**1.84**	Nascent protein folding; protein rescue	CTP
BN194_17470	Molecular chaperone (heat shock protein, Hsp70 complex)	GrpE	**0.38** **1.18**	-0.11 0.95	**1.70** **-1.05**[d]	Nascent protein folding	CTP
BN194_19060*	Foldase protein prsA	PrsA	**-0.05**	1.91	**2.02**	Protein secretion: exported protein folding	CMP
BN194_23750	Class I heat-shock protein (chaperonin) large subunit	GroEL	**0.62** **0.99** **0.06**	0.58 1.10 0.71	**3.61** **1.71**[c] **3.13**[d]	Nascent protein folding	CTP
BN194_23760	Class I heat-shock protein (chaperonin) small subunit	GroES	**0.81** **1.01**	0.6 0.88	**3.14** **1.53**[c]	Nascent protein folding	CTP
BN194_26400	Chaperonin (heat shock protein Hsp33)	HslO	**0.01**	0.00	**2.09**	Protein folding; stress response	CTP
BN194_26460	ATP-dependent zinc metalloprotease FtsH	FtsH	**-0.17**	1.78	**0.74**	Membrane protein assembly/recycling	CMP
BN194_29440	Alpha-crystallin domain acid shock protein, HSP20 family	BN194_29440,	**3.1**	5.39	**7.17**	Protein disaggregation (during rapid growth)	CWP
Proteases and protein recycling							
BN194_10510	ATP-dependent Clp protease proteolytic subunit	ClpP2	**0.14** **0.59**[d]	-0.16 1.57[d]	**1.07** **5.50**[d]	Recycling defective proteins	CTP
BN194_15340	ATP-dependent Clp protease ATP-binding subunit ClpX	ClpX	**0.12**	0.07	**-0.57**	Recycling defective proteins	CMP
BN194_15500	Chaperone protein ClpB	ClpB	**-0.14** **0.58** **-0.18**	-0.31 0.66 0.99	**1.34** **1.03**[c] **2.52**[d]	Recycling defective proteins	CTP
BN194_15880	Carboxy-terminal processing proteinase[c]	CtpA	**-0.15**	-0.34	**-0.41**	Protein activation; recycling defective proteins	IMP
BN194_16000	ATP-dependent protease, peptidase subunit	HslV	**-0.80** **0.42**[c]	-0.13 0.32[c]	**0.40** **0.88**[c]	Recycling defective proteins	CWP
BN194_16010	ATP-dependent protease ATPase subunit HslU	HslU	**-0.24** **0.00**	-0.26 0.85[d]	**-0.73** **2.0**[d]	Recycling defective proteins	CTP
BN194_19450	ATP-dependent Clp protease ATP-binding subunit ClpE	ClpE	**0.24** **-0.89**	0.22 1.08	**2.16** **2.99**[d]	Recycling defective proteins	CMP
BN194_19460	ATP-dependent chaperone/Clp protease	ClpA/B	**0.12**	0.06	**2.14**	Recycling defective proteins	CMP
BN194_21680	ATP-dependent Clp protease ATP-binding subunit	ClpC	**0.80** **0.62**	1.2 1.14	**3.12** **2.84**[d]	Recycling defective proteins	CMP

BN194_26550	Peptidyl-tRNA hydrolase	Pth	**0.54** **0.13**	0.49 -0.03	**0.15** **2.00**[c]	Defective peptide removal from tRNA	CTP
BN194_26350	Clp endopeptidase ATP-binding subunit	ClpB2	**-0.04**	-0.23	**-0.56**	Recycling defective proteins	CMP
BN194_29460	Heat-shock protein serine protease	HtrA/DegP	**0.29** **0.17** **3.32**	1.42 0.23 4.90	**2.61** **2.45**[c] **3.05**[d]	Recycling defective proteins; protein folding; stress response	IMP
DNA-directed RNA polymerase subunits and sigma factors							
BN194_03970	Sigma-54	RpoN/SigL	**ND**	ND	**ND**	RNA polymerase	CTP
BN194_04640	Sigma-24-like	BN194_04640	**ND**	ND	**ND**	RNA polymerase	CTP
BN194_09020	Sporulation-specific sigma factor	ComS/SigH	**ND**	ND	**ND**	RNA polymerase	CTP
BN194_13660	Subunit beta	BN194_13660 (EpuA)	**ND**	ND	**ND**	RNA polymerase	CMP
BN194_15030	Subunit epsilon	UPF0356-protein	**0.36**	0.42	**-0.58**	RNA polymerase	CTP
BN194_16920	Sigma factor A/Sigma-70	RpoD/SigA	**1.06**	0.55	**1.14**	RNA polymerase	CTP
No equivalent	Sigma factor A (2)/Sigma-70	RpoD/SigA				RNA polymerase	
BN194_18100	Subunit omega	RpoZ	**0.58**	-0.32	**-1.47**	RNA polymerase	CTP
BN194_25960	Subunit alpha	RpoA	**0.18** **0.85**[d]	0.20 1.79[d]	**1.55** **3.04**[d]	RNA polymerase	CTP
BN194_26330	Subunit beta'	RpoC	**0.17**	-0.04	**-0.40**	RNA polymerase	CTP
BN194_26340	Subunit beta	RpoB	**0.14** **0.60**[d]	-0.09 -0.61[d]	**-0.54** **2.07**[d]	RNA polymerase	CTP
BN194_26810	Subunit delta	RpoE	**0.25** **0**[d]	0.19 0[d]	**1.31** **2.10**[d]	RNA polymerase	CTP

[a] Log_2-transformed two-sample-*t*-test difference of the label-free quantification (LFQ) intensities for proteins in CFEs following culture at different temperatures relative to the control (30°C) from 3 technical replicates. LFQ values were considered significant at log_2-fold threshold of ≥1 (upregulation) or ≤-1 (downregulation), FDR <5%. ND = Not Detected

[b] Protein subcellular localization was assigned according to subcellular localization prediction tools: integral membrane proteins (IMP) were predicted using TMHMM server 2.0 algorithm (Krogh *et al*. 2001). Cytoplasmic proteins (CTP) and cell membrane-anchored proteins (CMP) were predicted by the cell-Ploc 2.0 (Chou & Shen 2010) Cell wall-associated proteins (CWP) were predicted by PSORTb (Nancy *et al*. 2010).

[c] Log_2 differences for lithium chloride fractions

[d] Log_2 differences for trypsin shaving fractions

[*] Signal peptides was determined using SignalP 4.1 server with Gram-positive bacteria selected as organism group (Petersen *et al*. 2011).

Table 6. 2. Orthologs of proteins regulated by Sigma-B in other Gram-positive bacteria

Gene locus	Protein name[a]	Protein symbol	35°C[b]	40°C	45°C	Function[a]	Predicted localization[c]	References
			Log_2 Ratio					
BN194_01950	Alkaline shock protein 23 family protein	Asp23_2	**ND**	ND	**	Cell envelope-related	CTP	(Müller *et al.* 2014)
BN194_17970	Uncharacterized protein Spy1614, Asp23 domain	YloU	**0.30**	0.25	**0.21**[d]	General stress protein (cell envelope-related?)	CWP	(Müller *et al.* 2014)
BN194_18230	Uncharacterized protein Spy1614, Asp23 domain	YqhY	**0.32**	1.28	**0.07**	Unknown (cell envelope-related?)	CWP	(Müller *et al.* 2014)
BN194_24800	CsbD superfamily protein	YhjA	**-0.40**	ND	**4.04**[d]	General stress response (phosphate starvation?)	CWP	(Prágai & Harwood 2002)
BN194_10150	Ribosome-associated sigma 54 modulation protein	Hpf	**-0.10**	0.23	**-1.99**	Ribosome-associated (stabilisation); attenuates translation	CTP	(Ueta *et al.* 2008)
BN194_28350	Uncharacterized protein, DgaF, aldolase domain	BN194_28350	**0.73** **0.77**	0.95 0.91	**2.41** **2.54**[d]	Unknown (D-glucosaminate metabolism?)	CWP	(Miller *et al.* 2013)

[a] Protein name and function from domains identified in UniProt

[b] Log_2-transformed two-sample-*t* test difference of the label-free quantification (LFQ) intensities for CFE fractions, unless otherwise annotated; ND = Not Detected

[c] Protein subcellular localization were determined as previously described in Table 6. 1

[d] Log_2 differences for lithium chloride fractions

** Detected only at the growth temperature in moderately high abundance

Table 6. 3. Differential modulation of proteins that are targets of the regulators in *L. casei* GCRL163 under prolonged heat stress.

Gene locus	Protein name	Protein symbol	35°C[a]	40°C	45°C	Function	Predicted localization[b]
			Log$_2$ Ratio				
Signal transduction							
BN194_02120	Cell wall integrity sensing response regulator CesR	CesR/VanR	**-0.05**	-0.67	**-0.52**	Cell wall alteration signalling	CTP
BN194_02130	Cell wall integrity sensing histidine kinase CesK	VanS	**-0.33**	-0.39	**-0.73**[c]	Cell wall alteration signalling	IMP
BN194_08710	Divergent methyl-accepting chemotaxis-like domain, DUF948 superfamily	BN194_08710	**0.53**	1.26	**0.87**	Chemotaxis?	IMP
BN194_10220	Phosphosensor response regulator	PhoP	**0.50**	0.37	**-2.79**	Phosphosensor	CTP
BN194_11790	Two-component system response regulator	BN194_11790	**-0.46**	-0.48	**-1.19**	Unknown signal transduction	CTP
BN194_13870	Universal stress protein, UspA superfamily	BN194_13870	**-0.23**	1.90	**1.07**	Stress regulation?	CTP
BN194_14450	GAF domain-containing protein	YtsP	**0.50**	-0.30	**ND**	Unknown signal transduction	CTP
BN194_17830	Regulator of the SRP pathway, sigma70_r4 superfamily	YlxM	**0.61**	-0.21	**-0.54**	Protein secretion; SRP pathway	CMP
BN194_18490	Cell wall stress sensing response regulator	LiaR	**-0.81**	-1.88	**-2.26**	Cell wall stress signal transduction?	CTP
BN194_18650	Response regulator ArlR	ArlR	**-0.29**	-0.37	**-0.92**	Oxidative stress sensing?	CTP
BN194_19250	Two-component system response regulator	BceR_2	**0.73**	1.17	**-0.23**	Unknown signal transduction	CTP
BN194_23500	Universal stress protein, UspA superfamily	BN194_23500	**ND**	ND	**ND**	Stress regulation?	CTP
BN194_29510	Two-component system response regulator	YycF_2	**0.23** **0.30**	0.09 0.35	**1.13** **1.29**[c]	Unknown signal transduction (fatty acid and cell wall hydrolase regulation?)	CTP
Transcriptional regulation							
BN194_02540	LytR family cell envelope-related function transcriptional attenuator	LytR	**0.43** **1.98**	1.17 1.83	**0.96** **0**[d]	Unknown regulation	CWP
BN194_02570	HTH-type transcriptional repressor glcR	GlcR	**-0.14**	-0.64	**-0.71**	Regulation of glycosyltransfer?	CTP
BN194_03780	HTH-type transcriptional regulator galR	GalR	**0.22**	-0.49	**-0.11**	Regulatory role of sugar uptake	CTP
BN194_03960	Transcriptional antiterminator with sigma54 interaction domain	CelR	**ND**	ND	**ND**	Regulation of sugar uptake PTS system genes?	CMP
BN194_05600	HTH_XRE family transcriptional regulator	BN194_05600	**0.50** **0.68**	0.10 0.65	**0.27** **1.00**[c]	Regulatory role unknown	CWP
BN194_08230	MarR family transcriptional regulator	BN194_08230	**0.15**	-0.34	**-1.65**	Regulatory role unknown	CTP
BN194_08410	Iron-dependent transcriptional regulator, DtxR family	BN194_08410	**-0.05**	-0.58	**0.83**	Regulation of iron uptake	CTP
BN194_08440	GntR family transcriptional regulator with CBS domains	YtoI	**-0.16**	-0.55	**-2.12**	Regulatory role unknown	CTP
BN194_08730	NAD biosynthesis repressor, HTH_11/3M domain superfamily	NiaR	**0.08**	-0.08	**1.25**	Regulation of de novo NAD (P)H synthesis	IMP
BN194_08750	Catabolite control protein A	CcpA	**0.08** **-1.23**	-0.44 0.61	**0.72** **-0.93**[d]	Regulation of catabolic enzyme coding genes	CTP
BN194_09330	phage HTH_XRE /HipB family transcriptional regulator	BN194_09330	**0.14** **0.80**	-0.10 1.00	**-0.21** **1.47**[c]	Phage associated protein (addiction module?)	CTP

BN194_10050	HTH_XRE transcriptional regulator	BN194_10050	0.37	2.14	2.35	Regulatory role unknown	CMP
BN194_10290	Phosphate transport system regulatory protein	PhoU	-0.52	0.56	0.52	Regulation of phosphate uptake genes	CTP
BN194_10500	Organic hydroperoxide resistance transcriptional regulator	OhrR	0.41	0.35	0.05	Regulation of hydroperoxidase genes	CTP
BN194_11420	Double-stranded beta-helix related protein	BN194_11420	-0.51	-1.16	ND	Regulatory role (polyamine-associated?)	CTP
BN194_11600	DNA-binding regulatory protein, YebC/PmpR family	BN194_11600	-0.10 -1.61	-1.25 -1.45	-0.66 -3.70[d]	Regulatory role (putative proteolytic system regulator)	CTP
BN194_12190	TetR family transcriptional regulator	BN194_12190	-0.17	-1.14	-1.75	Regulatory role unknown	CMP
BN194_13860	HTH_XRE family transcriptional regulator	BN194_13860	0.79 0.95	1.76 0.93	-2.29 -0.02[c]	Unknown regulation	CMP
BN194_16100	LysR family transcriptional regulator	BN194_16100	0.09	-1.17	-0.96	Unknown regulation	CTP
BN194_16620	Transcriptional regulator GltC	Nac	0.68	1.37	0.94	Regulatory role unknown	CTP
BN194_18590	Transcriptional regulator, HxlR family	YodB	ND 0.01	ND -0.16	ND 3.54[c]	Regulatory role unknown	CTP
BN194_18900	Ribonucleotide reductase regulator	NrdR	ND -0.38	ND -0.15	ND -0.87[c]	Regulation of ribonucleotide reductases	CTP
BN194_19880	GntR family transcriptional regulator	YvoA	-0.16	-0.28	0.05	Regulatory role unknown	CTP
BN194_21700	Transcriptional regulator, LytR_CpsA-Psr superfamily	LytR_3	0.51 -0.09	0.90 -0.21	-1.98 -2.02[c]	Regulatory role unknown	CWP
BN194_22670	MarR family transcriptional regulator	BN194_22670	0.09	0.37	1.45	Regulatory role unknown	CTP
BN194_22710	ArsR family transcriptional regulator	BN194_22710	0.26	0.85	2.88	Regulation of efflux?	CTP
BN194_23510	XRE family transcriptional regulator	BN194_23510	0.05 -0.74	-0.42 -0.62	1.13 -1.71[d]	Regulatory role unknown	CMP
BN194_23780	LytR_CpsA family transcriptional regulator	LytR_4	ND 1.06	ND 0.20	ND -2.37[c]	Regulatory role unknown	CWP
BN194_23820	Redox (NADH)-sensing transcriptional repressor	Rex	0.11	0.13	-0.84	Regulates genes associated with. fermentation; NADH:NAD sensor	CTP
BN194_24500	RpiR family transcriptional regulator	BN194_24500	-0.04	0.69	1.47	Regulation of carbohydrate metabolism genes	CTP
BN194_25900	MerR family transcriptional regulator	BN194_25900	0.42	-1.36	-2.94	Regulation of drug resistance transporter	CTP
BN194_26580	PadR family transcriptional regulator	BN194_26580	0.21	0.10	0.18	Regulatory role unknown	CWP
BN194_26960	Pur operon repressor	PurR_2	-0.27 0.00	-1.64 0.63	-1.57 -1.18[d]	Regulation of purine biosynthesis genes	CTP
BN194_28690	Glucitol operon repressor, DeoR family	SrlR	-0.06	0.21	-0.24	Regulatory role (carbohydrate-related?)	CTP
BN194_30170	HTH_XRE family transcriptional regulator	BN194_30170	0.08	-0.90	0.24	Unknown regulation	CWP
Transcription-associated proteins							
BN194_06990	Cold shock protein 1	Csp	-0.05 -0.92	-1.86 -4.61	-2.72 -5.47[d]	RNA chaperone	CTP
BN194_12460	Cold shock-like protein	CspLA	-0.08	-2.41	-3.00	RNA chaperone	CTP
BN194_17580	Transcription elongation protein	NusA	-0.10 0.80	-0.53 0.33	0.28 -1.47[d]	Transcription elongation	CTP
BN194_18220	Transcription termination factor	NusB	0.40	-2.26	-3.65	Transcription antitermination	CTP
BN194_18520	Transcription elongation factor GreA	GreA	0.11 0.42	-0.57 0.27	-1.71 1.49[c]	Transcription elongation	CTP
BN194_24260	Transcription antitermination protein	NusG	0.09 0.00	-0.64 0.00	0.10 1.57[d]	Transcription antitermination	CTP
DNA replication-related proteins							

BN194_00010	Chromosomal replication initiator protein	DnaA	-0.08	-0.70	-1.03	DNA replication initiation	CTP
BN194_00020	DNA polymerase III subunit beta	DnaN	0.46 0.00	0.18 0.00	0.31 2.17[d]	DNA replication elongation	CTP
BN194_00060	DNA gyrase subunit B	GyrA	0.29 -1.04	-0.32 -1.13	-1.08 -0.75[d]	DNA topological change	CTP
BN194_00070	DNA gyrase subunit A	GyrB	-0.10	-0.57	0.97	DNA topological change	CTP
BN194_00110	ssDNA-binding protein	Ssb	0.53	1.28	0.14	DNA replication initiation; DNA repair (homologous recombination)	CTP
BN194_01140	Replicative DNA helicase	DnaC	0.40	1.16	1.84	DNA replication initiation	CTP
BN194_11930	DNA ligase	LigA	0.11	-2.36	-4.84	DNA replication elongation (duplex ligation); DNA repair (double-stranded breaks)	CTP
BN194_13900	Recombination factor Holliday junction-associated protein	YrvN	0.64	0.12	0.00	DNA replication (Holliday junction)	CTP
BN194_15440	Checkpoint for chromosome synthesis GTP binding protein	Obg	-0.27	-0.47	-1.58	Regulate replication via DnaA; growth responsive); ribosome assembly; regulates (p)ppGpp during exponential growth	CTP
BN194_15740	DNA-binding protein HU	Hup/Hbs	-0.18 0.00 1.31	-0.20 0.00 1.76	0.29 2.00[c] 2.50[d]	DNA topology change	CTP
BN194_15970	DNA topoisomerase 1	TopA	0.79	-1.10	0.52	DNA topological change	CTP
BN194_16050	DNA topoisomerase 4 subunit B	ParE	0.32	-0.68	-1.11	DNA topological change	CTP
BN194_23990	DNA polymerase III subunit gamma/tau	DnaX	0.51	0.78	-2.48	DNA replication elongation	CTP
DNA repair/recombination							
BN194_07970	SOS response repressor (regulates RecA)	LexA	0.52	-0.44	ND	DNA repair (homologous recombination; SOS response)	CTP
BN194_08560	Recombination and DNA strand exchange inhibitor	MutS2	0.76	-0.46	-2.57	DNA repair (mismatch)	CTP
BN194_10440	Excinuclease ATPase subunit B	UvrB	0.40	1.87	-0.33	DNA repair (Uvr excision repair complex; SOS response)	CTP
BN194_10450	Excinuclease ATPase subunit A	UvrA	-0.16	0.53	0.78	DNA repair (Uvr excision repair complex; SOS response)	CTP
BN194_11680	Adenine-specific DNA methylase	YtxK	0.14	-0.25	-0.74	DNA repair-related	CTP
BN194_11920	ATP-dependent DNA helicase	PcrA	-0.02	-1.31	-1.94	DNA repair (Uvr excision repair complex; mismatch; SOS response)	CTP
BN194_18930	DNA polymerase I	PolA	-0.23	-2.26	-3.41	DNA repair (multiple roles)	CTP
BN194_21130	DNA helicase IV	HelD	0.06 0.01	-0.38 -0.04	-0.60 -1.18[c]	Nucleotide excision, mismatch repair	CTP
BN194_23720	Recombination and DNA strand exchange inhibitor	MutS	0.23	-2.26	-4.25	DNA repair (mismatch)	CTP
BN194_23970	Recombinational DNA repair protein	RecR	-0.06	-1.06	-0.32	DNA repair (homologous recombination)	CTP
BN194_27650	DNA recombinase RecA	RecA	0.36 0.17	0.14 2.35	-2.54 2.52[d]	DNA repair (homologous recombination; SOS response)	CTP
Protein translation							
BN194_02070	GTP-and nucleic acid- binding protein	EngD	0.51	0.61	0.24	translation attenuation?	CTP
BN194_10180	Peptide chain release factor 2	PrfB	0.16	0.38	2.34	Protein synthesis; translation release	CTP
BN194_11110	SsrA-binding protein	SmpB	0.67 0.07	0.46 0.27	1.18 1.50[c]	Rescuing stalled ribosomes	CTP
BN194_13510	Peptide chain release factor 1	PrfA	-0.09	-0.93	0.88	Protein synthesis; translation release	CTP
BN194_15310	Elongation factor Tu	Tuf	-0.03 0.80	-0.08 1.65	1.60 1.26[d]	Protein synthesis; translation elongation	CTP

BN194_17550	Translation initiation factor IF-2	InfB	**0.06** **-0.38** **-0.45**	0.33 -0.44 -1.23	**0.03** **-1.13**[c] **-3.48**[d]	Protein synthesis; translation initiation	CTP
BN194_17670	Elongation factor Ts	Tsf	**0.20** **0.81**	0.47 2.06	**2.08** **2.44**[d]	Protein synthesis; translation elongation	CTP
BN194_18240	Elongation factor P	Efp	**-0.03**	-0.04	**0.40**	Protein synthesis; translation elongation	CTP
BN194_18820	Translation initiation factor IF-3	InfC	**0.50** **0.25** **0.00**	0.85 0.11 0.06	**0.39** **1.87**[c] **2.28**[d]	Protein synthesis; translation initiation	CTP
BN194_19480	Peptide chain release factor 3	PrfC	**0.24**	-0.28	**0.54**	Protein synthesis; translation release	CTP
BN194_22050	Elongation factor P	Efp	**-0.36**	-0.64	**-1.34**	Protein synthesis; translation elongation	CTP
BN194_26000	Translation initiation factor IF-1	InfA	**-0.34** **0.39**	-0.12 1.06	**-0.94** **1.43**[d]	Protein synthesis; translation initiation	CTP
BN194_26270	Elongation factor G	Fus	**-0.04** **0.41**	-0.12 0.85	**1.07** **2.35**[d]	Protein synthesis; translation elongation	CTP
Ribosomal proteins							
BN194_00100	30S ribosomal protein S6	RpsF	**0.30**	0.50	**-0.08**	30S (SSU) ribosome	CTP
BN194_00120	30S ribosomal protein S18	RpsR	**0.27** **0.00**	0.61 0.00	**1.04** **1.79**[d]	30S (SSU) ribosome	CMP
BN194_01130	50S ribosomal protein L9	RplI	**0.54**	0.11	**-1.23**	50S (LSU) ribosome	CTP
BN194_14440	30S ribosomal protein S4	RpsD	**0.20** **0.42**	0.30 -0.78	**-0.26** **-1.34**[d]	30S (SSU) ribosome	CTP
BN194_15250	30S ribosomal protein S20	RpsT	**0.45**	0.44	**0.39**	30S (SSU) ribosome	CTP
BN194_15260	30S ribosomal protein S15	RpsO	**0.17**	-0.05	**0.17**	30S (SSU) ribosome	CTP
BN194_15720	30S ribosomal protein S1	RpsA	**0.08**	0.25	**1.32**	30S (SSU) ribosome	CTP
BN194_18290	50S ribosomal protein L21	RplU	**0.09**	-0.17	**-0.57**	50S (LSU) ribosome	CTP
BN194_18800	50S ribosomal protein L20	RplT	**0.77** **0.00**	0.89 0.00	**0.17** **3.29**[d]	50S (LSU) ribosome	CMP
BN194_24090	50S ribosomal protein L10	RplJ	**0.30** **-0.79**	0.65 2.25	**0.77** **4.01**[d]	50S (LSU) ribosome	CTP
BN194_24150	50S ribosomal protein L1	RplA	**0.18** **0.11**	0.49 -1.16	**-0.84** **0.23**[d]	50S (LSU) ribosome	CTP
BN194_24160	50S ribosomal protein L11	RplK	**0.21** **-0.07** **2.33**	0.33 0.09 1.49	**-0.17** **1.10**[c] **1.32**[d]	50S (LSU) ribosome	CTP
BN194_25970	30S ribosomal protein S11	RpsK	**0.55** **-0.08**	0.57 2.58	**0.23** **1.87**[d]	30S (SSU) ribosome	CMP
BN194_25980	30S ribosomal protein S13	RpsM	**0.25**	0.65	**0.62**	30S (SSU) ribosome	CTP
BN194_26030	50S ribosomal protein L15	RplO	**0.43** **0.53**	0.71 3.22	**-0.11** **3.81**[d]	50S (LSU) ribosome	CTP
BN194_26050	30S ribosomal protein S5	RpsE	**0.40** **-1.11**	0.40 0.25	**0.04** **0.94**[d]	30S (SSU) ribosome	CTP
BN194_26060	50S ribosomal protein L18	RplR	**0.13** **1.59**	0.32 0.77	**-0.63** **-0.31**[d]	50S (LSU) ribosome	CTP
BN194_26070	50S ribosomal protein L6	RplF	**0.44** **0.60**	0.49 -0.71	**-1.03** **2.85**[d]	50S (LSU) ribosome	CTP
BN194_26080	30S ribosomal protein S8	RpsH	**0.21**	0.30	**-0.37**	30S (SSU) ribosome	CTP
BN194_26090	30S ribosomal protein S14 type Z	RpsZ	**0.59**	0.41	**-0.64**	30S (SSU) ribosome	CTP
BN194_26100	50S ribosomal protein L5	RplE	**0.31** **-0.28**	0.63 -0.66	**-0.14** **2.82**[d]	50S (LSU) ribosome	CTP
BN194_26110	50S ribosomal protein L24	RpsX	**0.72** **-0.09** **2.04**	0.75 -0.33 4.07	**-0.82** **2.04**[c] **1.40**[d]	50S (LSU) ribosome	CTP
BN194_26120	50S ribosomal protein L14	RplN	**0.37**	0.42	**-0.80**	50S (LSU) ribosome	CTP
BN194_26130	30S ribosomal protein S17	RpsQ	**0.09**	0.44	**0.43**	30S (SSU) ribosome	CTP
BN194_26150	50S ribosomal protein L16	RplP	**0.47**	0.40	**-0.46**	50S (LSU) ribosome	CTP
BN194_26160	30S ribosomal protein S3	RpsC	**0.27** **-0.32**	0.17 -1.18	**-0.49** **-1.54**[d]	30S (SSU) ribosome	CTP
BN194_26170	50S ribosomal protein L22	RplV	**0.34**	0.54	**-0.30**	50S (LSU) ribosome	CTP

			-1.62	1.85	1.53[d]		
BN194_26180	30S ribosomal protein S19	RpsS	0.23	0.39	-0.59	30S (SSU) ribosome	CTP
			0.00	-0.49	1.88[d]		
BN194_26190	50S ribosomal protein L2	RplB	0.51	0.68	-0.47	50S (LSU) ribosome	CWP
			0.94	0.91	1.56[d]		
BN194_26200	50S ribosomal protein L23	RplW	0.72	0.97	-0.09	50S (LSU) ribosome	CTP
			-0.23	-0.29	1.31[c]		
			5.41	1.34	5.41[d]		
BN194_26210	50S ribosomal protein L4	RplD	0.31	0.60	-0.53	50S (LSU) ribosome	CMP
			0.95	0.21	4.37[d]		
BN194_26220	50S ribosomal protein L3	RplC	0.21	0.37	-0.54	50S (LSU) ribosome	CTP
BN194_26230	30S ribosomal protein S10	RpsJ	0.30	0.61	-0.54	30S (SSU) ribosome	CTP
			0.19	-1.59	0.94[d]		
BN194_26280	30S ribosomal protein S7	RpsG	0.23	0.43	-0.25	30S (SSU) ribosome	CTP
			-0.95	-1.80	0.94[d]		
BN194_26290	30S ribosomal protein S12	RplL	0.40	0.76	0.18	30S (SSU) ribosome	CTP
tRNA/ribosome assembly/processing							
BN194_02020	Ribosomal RNA small subunit methyltransferase G	RsmG	0.37	0.34	1.37[c]	Ribosomal RNA processing (SSU)	CTP
BN194_09990	tRNA (cytidine (34)-2'-O)-methyltransferase	TrmL	-0.32	-0.88	0.17	tRNA modification	CTP
BN194_11100	Ribonuclease R	Rnr	0.39	-0.48	-1.85	Ribosomal RNA processing	CTP
BN194_11350	YjeE family protein	YdiB/TsaE	ND	ND	** [c]	tRNA threonyl carbamoyladenosine modification	CTP
BN194_14480	Thiamine biosyn. ATP pyrophosphatase/tRNA sulfurtransferase	ThiI	-0.15	-0.16	-0.79	tRNA modification; thiamine biosynthesis	CTP
			ND	-0.26	ND[c]		
BN194_14930	tRNA-specific 2-thiouridylase	MnmA	-0.15	0.08	-2.81	tRNA modification	CTP
BN194_15770	tRNA CCA-pyrophosphorylase	Cca	0.19	1.51	-0.58	tRNA modification	CTP
BN194_15940	Ribosome biogenesis GTPase A	RbgA	0.73	1.20	0.68	50S ribosomal subunit assembly	CTP
BN194_16670	RNA-binding methyltransferase	YpsC	-0.13	-1.79	-1.21	Ribosomal RNA acetylation?	CTP
BN194_16850	Ribosomal RNA small subunit methyltransferase F	RsmF	1.46	0.59	-0.69	Ribosomal RNA processing (SSU)	CTP
BN194_17870	Ribonuclease III	Rnc	0.89	0.90	-1.64	Ribosomal RNA processing (SSU/LSU)	CTP
BN194_18610	23S rRNA methyltransferase, SpoU_sub_bind/SpoU_methylase superfamily	YsgA	0.92	0.67	-0.49	Ribosomal RNA processing (LSU)	CTP
BN194_18970	tRNA-binding protein, tRNA_domain_binding superfamily	YtpR	0.30	-0.62	-3.82	Unknown (tRNA-associated?)	CTP
BN194_24420	TrmH family tRNA/rRNA methyltransferase	YacO	0.18	0.61	-0.42	Ribosomal RNA processing (LSU)	CTP
BN194_26500	RNA binding protein, S1_like superfamily	YabR	0.37	-1.08	-1.48	Unknown ribosome-associated function	CTP
			0.47	-1.13	-2.94[d]		
BN194_26520	Ribosome-associated heat shock protein implicated in the recycling of the 50S subunit (Hsp15)	YabO	1.27	0.89	-1.54	Ribosome recycling (LSU); stress response?	CTP
			-0.04	-0.26	2.49[c]		
BN194_27020	Ribosomal RNA small subunit methyltransferase A	RsmA	0.53	-0.60	-1.52	Ribosomal RNA processing (SSU)	CTP
BN194_30600	tRNA modification GTPase	MnmE	-0.02	-0.01	1.48	tRNA modification	CTP
BN194_30620	Ribonuclease P protein component	RnpA	0.68	1.45	-0.63	tRNA and 4.5S processing	CMP
			0.90	-0.16	1.24[c]		
tRNA aminoacyl synthesis							
BN194_08480	Alanine--tRNA ligase	AlaS	-0.11	-0.79	-1.29	Protein synthesis; tRNA charging	CTP
			-0.04	0.01	1.92[d]		
BN194_18560	Phenylalanine--tRNA ligase subunit beta	PheS	0.12	-0.54	-2.17	Protein synthesis; tRNA charging	CTP
BN194_18570	Phenylalanine--tRNA ligase	PheT	0.11	-0.21	-1.60	Protein synthesis; tRNA	CTP

	subunit alpha					charging	
BN194_24470	Glutamate--tRNA ligase	GltX	**-0.16**	-0.90	**-2.43**	Protein synthesis; tRNA charging	CTP
BN194_27130	Methionine--tRNA ligase	MetS	**0.31**	-0.10	**0.59**	Protein synthesis; tRNA charging	CTP
RNA degradation							
BN194_10100	Endoribonuclease (Rnase Y)	Rny	**-0.53**	2.44	**0.76**	mRNA decay (riboswitch turnover)	CTP
BN194_15020	Ribonuclease J1	RnjA	**0.14**	-1.14	**1.41**	RNA degradation (RNA degradosome)	CTP
BN194_15290	Ribonuclease J2	RnjB	**-0.18**	1.07	**1.57**	RNA degradation (RNA degradosome)	CTP
			0.00	0.00	**2.58**[d]		
Post-translational modification							
BN194_08570	Thioredoxin	TrxA_2	**0.09**	-0.17	**-5.18**	Protein Cys residue reduction	CWP
			-0.05	-0.22	**1.42**[c]		
BN194_10360	Thioredoxin reductase	TrxB	**0.47**	0.69	**1.37**	Thioredoxin reduction	CMP
BN194_17060	Phosphotransferase activator of gluconeogenesis	Rp2	**0.10**	0.60	**-0.47**	Putative pyruvate, phosphate dikinase regulatory protein	CTP
BN194_18980	Thioredoxin family protein	YtpP	**0.19**	-0.22	**-3.68**	Protein Cys residue reduction	CWP
			0.47	0.07	**2.17**[c]		
BN194_23520	Tyrosine-protein phosphatase	Ptp3	**0.66**	-2.22	**-2.61**	Protein phosphorylation (regulation)	CWP
			-0.52	-0.32	**-2.12**[c]		
BN194_27190	NAD-dependent protein deacylase	CobB	**0.56**	0.94	**2.47**	Protein deacylase, sirtuin-type	
			0.46	0.66	**3.03**[c]		
BN194_29310	Protein-tyrosine/serine phosphatase, PTPc superfamily	BN194_29310	**0.41**	0.74	**-0.09**	Protein phosphorylation (regulation)?	CWP
			0.87	0.89	**2.05**[c]		
Cytokinesis							
BN194_02030	Chromosome partitioning (nucleoid occlusion) regulatory protein	Noc	**1.17**	0.27	**0.79**	Cell division (chromosomal segregation)	CTP
BN194_02050	Chromosome partitioning regulatory protein	ParB	**-0.01**	-0.25	**-0.76**	Cell division (chromosomal segregation)	CTP
BN194_08540	Cell division protein	BN194_08540	**0.40**	1.10	**0.84**	Z-ring protein bundle promoter, ZapA superfamily	CTP
BN194_14460	Septation ring formation regulator	EzrA	**0.12**	2.55	**-0.61**	Cell division; divisome complex	CMP
			0.15	0.49	**-1.13**[c]		
BN194_16690	Cell cycle initiation protein	GpsB	**0.28**	1.66	**2.52**	Cell division; divisome complex. Regulates PBP1 localization during cell cycle progression	CTP
BN194_17860	Chromosome condensation and segregation protein	Smc	**-0.22**	-1.36	**-3.36**	Cell division (chromosomal segregation)	CTP
Others							
BN194_00040	RNA-binding protein, S4_2 superfamily	YaaA	**-0.36**	-0.22	**-2.08**	Unknown (DNA replication and/or repair?)	CTP
BN194_08040	DUF448 superfamily protein	YeaO	**0.50**	0.07	**2.58**[c]	Unknown	CTP
BN194_10070	Competence damage induced protein, MoCF_BD/CinA superfamily	CinA	**-0.10**	1.19	**-1.12**	Unknown (DNA damage regulatory role?)	CTP
BN194_15600	RNA-binding protein, COG2996 superfamily	YitL	**-0.63**	-1.07	**ND**	CvfB_family, regulation of protein export?	CTP
BN194_17620	Zn-dependent metalloprotease similar to alternative sigma factor repressor antagonist	Eep	**ND**	ND	**ND**	Unknown (regulatory role?)	IMP
			-0.61	-0.31	**-1.46**[c]		
BN194_22340	Type I restriction modification system, M subunit	HsdM	**0.86**	-1.04	**-2.90**	Foreign DNA restriction	CTP
BN194_23980	YbaB/EbfC DNA-binding family protein	BN194_23980	**0.52**	0.46	**1.12**	Unknown	CTP
BN194_26610	CBS pair domain superfamily protein	BN194_26610	**0.43**	0.88	**1.33**	Unknown	CMP
			0.02	-0.31	**1.48**[c]		

BN194_27010	DUF1021 superfamily protein	BN194_27010	**ND*** **	ND	**ND**	Unknown	CWP

[a] Log_2-transformed two-sample-*t* test difference of the label-free quantification (LFQ) intensities
[b] Protein subcellular localization were determined as previously described in Table 6. 1.
[c] Log_2 differences for lithium chloride fractions
[d] Log_2 differences for trypsin shaving fractions
** Detected only at the growth temperature
*** Only detected at 30°C

CHAPTER 7

PROTEOMIC ANALYSIS OF *LACTOBACILLUS CASEI* GCRL163 UNVEILS RE-ROUTING OF METABOLIC PATHWAYS FOR ENERGY GENERATION AND UTILIZATION OF ALTERNATIVE CARBON SOURCES DURING PROLONGED HEAT STRESS

7.1 Abstract

Lactobacillus casei are beneficial food-associated lactic acid bacteria used industrially as starter cultures for dairy food fermentation or probiotics to boost health. Heat stress is a commonly encountered condition during these applications, and understanding the metabolic mechanisms underpinning the adaptation of the strain to prolonged heat stress is vital for improving bacterial functionalities. To investigate the modification of the metabolic pathways under prolonged heat stress, we analysed label-free quantitative proteomic datasets of cell free-extract, cell surface-associated (trypsin shaving and LiCl-sucrose extracts) and extracellular culture fluid protein fractions of *L. casei* GCRL163, cultured to mid-exponential growth phase at 30°C, 40°C and 45°C and pH 6.5 under controlled growth conditions. The proteomic data revealed upregulation of EIIBGal, Lev- and Man-family proteins, involved in PTS-uptake of sugars other than glucose, suggesting utilization of alternative sources of carbon during heat stress. Proteins GalE_2, GalT, BGAL17, Cap4C and BN194_07390, involved in galactose, fructose and sucrose metabolism were repressed. Networks of metabolic pathways were activated to channel carbon into the glycolytic pathway, including phospholipid metabolism coupled to up-regulated TpiA, pentose phosphate pathway shunt via upregulation of GntK and Gnd, tagatose metabolism by over-expressed PfkA, LacC, LacD2 and LacD2_2 and amino and nucleotide sugar metabolism by upregulation of NagA, ManD and NagB. Proteins involved in nucleotide metabolism, peptidoglycan biosynthesis

and high energy-requiring fatty acid biosynthesis were repressed at 45°C, while RNA degradation increased. This study demonstrates that a key mechanism of prolonged heat stress response in *Lactobacillus* spp. involves efficient management of energy generation and utilization, particularly around carbon scavenging pathways.

Keywords: Proteomics, *Lactobacillus casei*, metabolism, probiotic, heat stress.

7.2 Introduction

Lactobacillus species play primary roles in the manufacture of fermented food products and contribute to improved health in humans and livestock as probiotics (Angelis *et al*. 2016). Probiotics can act as preventive or bio-therapeutic agents in clinical diarrhoea (Szajewska & Mrukowicz 2005), rheumatoid arthritis (de los Angeles *et al*. 2011; Hatakka *et al*. 2003) and lactose intolerance (Reid 1999; Suarez *et al*. 1995; Vonk *et al*. 2012). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) indicate probiotics as "*live microorganisms which, when administrated in adequate amounts, confers a health benefit on the host*" (Hill *et al*. 2014). Furthermore, *Lactobacillus* spp. are extensively used in the industrial production of fermented food products, to facilitate the development of organoleptic and hygienic quality of fermented products such as fermented milk and cheese products (Mäyrä-Mäkinen & Bigret 1998). Notably, the mesophilic species *L. casei* contributes to flavour development in Cheddar cheese as a non-starter lactic acid bacterium (Fox *et al*. 1998). These bacteria are frequently exposed to stress, including high and low temperature, extremes in osmolality and pH, plus nutrient limitation (De Angelis *et al*. 2011). To survive, cells modulate gene expression resulting in altered metabolic pathways involved in proteolysis, carbohydrate and amino acid metabolism, which have pivotal roles in food biotechnology (Giraffa *et al*. 2010; Gobbetti *et al*. 2005). In industrial

processing systems, the application of heat stress can impact on beneficial bacteria such as *Lactobacillus* spp (De Angelis *et al*. 2011).

Metabolic processes, such as proteolysis, carbohydrate and amino acid metabolism, are pivotal in food biotechnology (Giraffa *et al*. 2010; Gobbetti *et al*. 2005; van de Guchte *et al*. 2002). Understanding how cells modulate their key metabolic pathways under prolonged heat stress is vital for boosting the current knowledge of PHSR in lactobacilli and obtaining high-quality strains with improved functionalities. Papadimitriou *et al*. (2016) suggested that selecting mutants within lactic acid bacteria populations through adaptive changes induced by experimental evolution, exemplified by prolonged exposure to stressing environments, will be important in strain improvement through non-genetically modified approaches.

Proteomic analyses have been used to study metabolic processes in several *Lactobacillus* species (Fernandez *et al*. 2008; McLeod *et al*. 2010; Pessione *et al*. 2010). Advances in proteomics from discovery-based analytical workflows to quantitative proteomics (Champomier-Vergès *et al*. 2002) can foster understanding of metabolic processes under stress conditions. In a previous study, we employed label-free quantitative (LFQ) proteomics to investigate PHSR of a Cheddar cheese isolate, *L. casei* GCRL163, resulting from prolonged exposure to supraoptimal growth-permissive temperatures in different growth fractions (Chapters 3 and 4). The CFE, LS and TS filtered data contained 859, 826 and 183 proteins respectively while the ECF data represented a filtered dataset of 47 proteins. Subcellular localization prediction analyses (Emanuelsson *et al*. 2000) of the LS, TS and ECF datasets further revealed a total of 416 surface proteins in *L. casei* GCRL163 (Chapter 4). The findings demonstrated that *L. casei* GCRL163 grown at 40°C modulated an array of proteins for cellular adaptation, including proteins associated with the transport of

molecules, molecular chaperones and other proteins involved in quality control systems and the maintenance of the cell envelope. Furthermore, at elevated growth temperature of 45°C, the bacterial cells repressed the expression of several proteins (Chapter 3). Several of these proteins were not only regulated in the CFE protein fractions, which represented largely cytoplasmic proteins, but also in the cell surface and ECF protein fractions of *L. casei* GCRL163 during prolonged heat stress (Chapter 4). To complement these findings, the current investigation provides an overview of the impact of prolonged heat stress on the key metabolic pathways in *L. casei* GCRL163 by examining protein expression in the CFE, surface-associated (LS and TS extracts) and ECF fractions obtained from *L. casei* GCRL163, cultured anaerobically at pH 6.5 and at 30°C, 40°C and 45°C in bioreactors under controlled growth conditions. The proteomic analysis demonstrated that the metabolic processes, involving the metabolism of protein synthesis, lipid and carbohydrates, were markedly impacted by prolonged heat stress in *L. casei* GCRL163.

7.3 Results

7.3.1 Prolonged heat stress led to upregulation of proteins involved in sugars other than glucose uptake in the phosphotransferase system

The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) is one of the main mechanisms through which carbohydrates are transported into cells in *L. casei* (Monedero *et al*. 2007). In this study, PtsH (BN194_19430) was repressed at 40°C and 45°C (although up-regulated at 45°C in the LS and at 40°C and 45°C in the TS), PtsI (BN194_19410) inhibited only at 45°C (also repressed in the LS at 45°C) but HprK (BN194_10330), detected only in the CFEs, was up-regulated at 45°C relative to 30°C in the CFEs (Figure 7. 1, Supplementary Table 7. 1 and see Supplementary Table 4.4). Protein

PtsG-1/Crr (BN194_28320) was not detected in any of the fractions despite culturing *L. casei* GCRL163 in glucose-rich MRS broth. However, BglP (BN194_06940), putatively associated with the uptake of β-glucoside, was highly up-regulated at 40°C and 45°C (log_2 2.5 and 4.0-fold respectively) in the CFEs but down-regulated at all the growth temperatures in the LS fraction. We also detected higher expression of some proteins, including LevG/ManZ (BN194_02990), LevF/ManY (BN194_02980) and ManZ_9 (BN194_29700) putatively associated with mannose, fructose or glucose uptake at 40°C and 45°C in the CFEs, although LevF/ManY was repressed at 45°C in the LS. Others such as LevE/ManX (BN194_02970) and ManX (BN194_29720, also repressed at 45°C in the LS and TS with upregulation at 35°C and 40°C) were abundantly expressed at 40°C but down-regulated at 45°C in the CFEs (Supplementary Table 7. 1 and see Supplementary Table 4.4). Furthermore, protein BN194_04820 was repressed at 40°C and 45°C in the CFEs. GmuB (BN194_23840) (oligo-beta-mannoside-specific phosphotransferase enzyme IIB component), with a lactose/cellobiose family domain, was only moderately enhanced at 45°C. Intriguingly, GmuB is located together with proteins putatively involved in tRNA biosynthesis in *L. casei* W56 (Hochwind *et al*. 2012). Protein MtlA (BN194_30410), putatively associated with mannitol uptake, was repressed at 45°C while proteins potentially performing uptake of sorbitol (SrlB, BN194_09940) or galactitol (BN194_27050) showed no change in relative abundance in the CFEs and LS. Overexpression of FruA_3/FruB (BN194_15410), associated with the PEP-fructose phosphotransferase system, at 40°C and 45°C was observed in the CFEs, at 45°C in the TS but was repressed at 35°C and 40°C in the LS. These findings suggested that the PTS play a vital role in PHSR. The data suggest *L. casei* GCRL163 cells attempts to transport carbohydrates which are not major constituents of the MRS broth, suggesting scavenging of endogenous carbohydrate sources from the yeast extract in the MRS broth and/or possibly exopolysaccharide (including peptidoglycan) degradation.

7.3.2 Impact of prolonged heat stress on carbohydrate metabolism

Several proteins involved in key metabolic processes and utilization of carbohydrates in *L. casei* GCRL163 were impacted by heat stress. These metabolic processes include glycolysis/gluconeogenesis, the pentose phosphate pathway and metabolism of glyoxylate, pyruvate, galactose, fructose and tagatose; and amino sugar and nucleotide sugar metabolism. Elevated growth temperature of 45°C led to upregulation of most glycolytic enzymes (PfkA, Fab_2, Gap, Gap_2, Pgk, GpmA2 and Eno) in the CFEs (Figure 7. 2, Supplementary Table 7. 1) and some of these proteins showed different pattern of expression in the cell surface fractions (see Supplementary Table 4.4). For instance, Fab_2 (BN194_05060) and Pgk (BN194_11020) were repressed in the TS, although Pgk showed differential abundance in the LS at 45°C. Pyk (BN194_15580) was repressed in the LS at 45°C, TS at 40°C and 45°C, and in the CFEs at 35°C. Many of these enzymes are highly abundant in cells and some have more than one copy in the genome, including Gap and Fba. Glycerone phosphate, which is a key substrate in glycerolipid and glycerophospholipid synthesis, can be converted to the glyceraldehyde-3P through a reaction involving TpiA. Although the enzyme TpiA was not differentially modulated at 40°C in the CFEs (up-regulated at 40°C in the TS), the elevated growth temperature of 45°C increased its expression in the CFEs and LS, suggesting a slight shift to catabolism.

Glycolysis is linked to other metabolic pathways, including the pentose phosphate (Entner-Doudoroff) pathway through reactions involving β-D-glucose, β-D-fructose-6P and glyceraldehyde-3P. In the Entner-Doudoroff pathway, GntK and Gnd are involved in the conversion of D-gluconate to D-gluconate-6P and then to D-ribulose-5P respectively in the proximal part of the metabolic pathway. The GntK (BN194_02340) in the CFEs and Gnd

(BN194_18660) in the CFEs, TS and LS were up-regulated at 45°C (Figure 7. 2, Supplementary Table 7. 1 and see Supplementary Table 4.4). The distal part of the pathway involves the reversible conversion of D-ribulose-5P to D-ribose-5P by RpiA_2 and then to phosphoribosyl pyrophosphate (PRPP) by Prs1. Prs1 (BN194_26930, moderately repressed at 40°C) and Prs2 (BN194_30370, repressed at 40°C and 45°C) in the CFEs. The reversible conversion of D-ribose-1P or D-ribose to D-ribose-5P involves DeoB or RbsK respectively. Both enzymes DeoB (BN194_02800) and RbsK (BN194_03300) were also repressed following prolonged heat stress at 40°C and 45°C in the CFEs. These results may suggest a shunting of the Entner-Doudoroff pathway at the level of D-ribulose-5P to the fructose-6P and glyceraldehyde-3P in the glycolytic pathway, while reducing the synthesis of phosphoribosyl pyrophosphate (PRPP), which is a precursor for purine, pyrimidine and histidine metabolism. Moreover, we also detected upregulation of XpkA (BN194_01710) , involved in the conversion of D-xylulose-5P to glyceraldehyde-3P, at 40°C (log_2 1.7-fold) and 45°C (log_2 2.3-fold) in the CFEs and was differentially abundant in the TS and LS protein fractions at these growth tempeartures. However, DeoC, involved in the reversible conversion of 2-deoxy-D-ribose-5P to glyceraldehyde-3P, was repressed at 40°C and 45°C growth temperatures relative to 30°C in the CFEs.

D-Galactose is metabolised via two pathways, the Leloir and tagatose-6P, which function in both lactose metabolism when this sugar is present and in normal cellular functions linked with exopolysaccharide synthesis involving nucleotide- and amino-sugars. For both pathways, the genes are clustered although not co-reguated but there are multiple copies of genes specifying proteins that perform similar functions in the *L. casei* genome. For example, surface-located BN194_16020 (aldose 1-epimerase) and galactose mutarotase (BN194_27070) putatively perform the same function as GalM, aldose 1-epimerase

(BN194_07390) in the Leloir pathway region. Proteins responsible for the uptake and phosphorylation of lactose and cleavage into β-D-galactose-6P (LacE, LacG and LacF_2) were not detected (Figure 7. 3). GalM was highly repressed at 45°C while BN194_16020 and BN194_27070 were highly up-regulated at this temperature in the CFEs (Supplementary Table 7. 1). The other Leloir operon proteins, GalK (BN194_07340), GalT (BN194_07360-70) and GalE (BN194_07350), were detected with similar abundance across all temperatures, while GalE_2 and Cap4C (BN194_12280) were highly down-regulated in CFEs, overall suggesting that the availability of UDP-glucose for the conversion of galactose into α-D-glucose-1P was lowered. The upregulation of galactose 1-epimerases and the galactose-specific PTS EIIB component, plus continued presence of GalK, suggest that carbon originating from galactose (and its acetylated and aminated derivatives) was preferentially moving through the tagatose-6-phosphate pathway following conversion to α-D-galactose-6P. This is supported by detecting a much greater abundance of LacC (BN194_07470), LacD2 (BN194_07480) (notably in LS and TS surface protein fractions) and LacD2_2 (BN194_27060) at 45°C, while LacA (BN194_07500) and LacB (BN194_07490) expression was not changed relative to the control in any cellular fraction (Supplementary Table 7. 1 and see Supplementary Table 4.4).

The gene specifying BGAL17 (β-galactosidase 17) in *L. casei* BL23 is part of the *gnb* operon, which includes the *agaS* (involved in N-acetyl-sugar catabolism), *manD* and 4 genes for PTS sugar transport (LevE and mannose/fructose/sorbose family transporters) which have been implicated in transporting N-acetyl-galactosamine (Bidart *et al*. 2014). BGAL17 (BN194_02960) was detected at low abundance in CFEs at all temperatures except 45°C and was not detected in LS, TS or ECF fractions although it is predicted to be an extracellular protein. Two of the 4 transporters (IID and EIIAB) were up-regulated at 40°C and/or 45°C.

We suggest that cell wall hydrolases, including cell wall hydrolaseBN194_02430 and other glycohydrolases in addition to the amidases and peptides detected as up-regulated at elevated temperatures, degrade the cell surface exopolysaccharides and peptidoglycan to liberate N-acetylated-amino sugars. The galactose-based sugars are then metabolised through the tagatose-6P pathway, channelling additional carbon sources to the glycolytic pathway through glyceraldehyde-3P at elevated growth temperatures for energy production.

Furthermore, α-D-glucose-1P can be converted to ADP-glucose by GlgD as the first, and rate limiting step, in synthesis of starch/glycogen storage carbohydrates. The protein GlgD (BN194_21590) was moderately up-regulated (log_2 1.2 and 1-fold) at 40°C and 45°C respectively in the CFEs (Figure 7. 3, Supplementary Table 7. 1), although the subsequent enzymes in this pathway (GlgABC) were not detected nor were GlgP (regenerates α-D-glucose-1P from starch), debranching amylase or maltase. However Agl (BN194_27950) was seen as more abundant, suggesting some turnover of storage glycogen reserves was used to generate glucose units. Agl contains domain structure that can be found in the maltogenic amylase and its ortholog is alph-1, 4-glucosidase (Consortium 2016). Moreover, ManD (BN194_02950) and NagA, associated with the synthesis of glucosamine-6-phosphate (GlcN-6P) from N-acetylglucosamine-6-phosphate (GlcNAc-6P), were up-regulated at 45°C in the CFEs. The protein NagB converts the GlcN-6P to fructose-6P with the reverse reaction catalyzed by GlmS. The protein NagB (BN194_30440) and GlmS (BN194_11560) were repressed at 40°C in the CFEs. Other proteins participating in uridine diphosphate (UDP) sugar metabolism, including GlmM, GlmU, MurA2 and MurB, were not differentially modulated at 40°C while GlmU (BN194_26940) and MurB (BN194_11400) were repressed (approximately log_2 1.0-fold) at 45°C in the CFE protein fraction. UDP-MurNAc formed from UDP-GlcNAc-enopyruvate by the MurB is a vital precursor in peptidoglycan

biosynthesis in the strain. The proximal part of the peptidoglycan biosynthesis in the cytoplasm, carried out by MurC, MurD, MurE, and MurG, was not impacted by thermal stress at 40°C in the CFE protein fraction (Figure 7. 4). However, MurF (BN194_26720) was repressed (log_2 1.2-fold) at 40°C while MurE (BN194_02160) and MurF were also repressed (log_2 1.3 and 1.8-fold respectively) at 45°C. The proteins PonA, PbpF, PbpF_2 and DacA are the main proteins involved in polymerization reactions during peptidoglycan biosynthesis. We found cell membrane-anchored protein PonA (BN194_16720) to be up-regulated in the TS (log_2 1-fold) and CFE (log_2 1.2-fold) at 40°C and less abundant at 45°C in the TS and LS protein fractions (Supplementary Table 7. 1 and see Supplementary Table 4.4). Also, DacA (BN194_02140), predicted to be cell membrane-anchored with N-terminal secretion motifs, was only enhanced (log_2 1-fold) at 40°C in the CFEs but repressed in the LS (log_2 1.9-fold), TS (log_2 1.9-fold) and CFEs (log_2 1.1-fold) protein fractions at 45°C. The integral membrane protein PbpF was inhibited at 45°C by log_2 1-fold while PbpF_2 showed no change in relative abundance in LS protein fraction. Both proteins were not differentially abundant at 40°C. However, PbpF (BN194_08770) and PbpF_2 (BN194_19090) were detected in the ECF protein fraction only at 40°C relative to 30°C (see Supplementary Table 4.4). These findings demonstrated that carbon source was further generated from the amino- and nucleotide-sugar metabolism into the glycolytic pathway via upregulation of NagA, NabB and ManD and proteins involved in synthesizing precursors for peptidoglycan biosynthesis were repressed. Alteration in cell surface integrity is supported by the observed greater fragility of cells at elevated temperatures during lithium chloride extraction and trypsin shaving (Chapter 4).

In pyruvate metabolism, both PdhA and PdhB catalyze the conversion of pyruvate to 2-hydroxyethyl-ThPP and then to S-acetyl-dihydrolipoamide-E. Three subunits of the

pyruvate dehydrogenase complex (PdhA, PdhB and PdhC) were all increased at elevated temperatures while PdhD was enhanced only at 40°C relative to 30°C in the CFEs (Figure 7. 5 and Supplementary Table 7. 1). The formation of acetyl-CoA from S-acetyl-dihydrolipoamide-E is catalyzed by PdhC while PdhD participates in the recycling-reaction, reversible conversion of dihydro-lipoamide to lipoamide-E. The formed acetyl-CoA may either feed into fatty acid or propanoate metabolism or is reversibly converted to acetyl-P or acetaldehyde then ethanol (see Chapter 3). The conversion of acetyl-CoA to acetyl-P involves the enzyme Pta while the formation of acetaldehyde involves Adh2/AdhE. The protein Adh2/AdhE (BN194_08400) was repressed (log_2 2.9-fold) while Pta (BN194_11340) showed no change in relative abundance at 40°C in the CFEs. Both enzymes Adh2/AdhE and Pta were up-regulated (log_2 3.5 and 1-fold respectively) at 45°C. Adh2/AdhE is also involved in the generation of ethanol as a fermentation end-product. Acetyl-P can also be generated from pyruvate by Pox5. The protein Pox5 (BN194_19670) was enhanced (log_2 1.4-fold) at 40°C and repressed (log_2 2.0-fold) at 45°C in the CFEs. Acetate is formed from acetyl-P by AckA, AckA_2 or AcyP. AckA (BN194_01620) was up-regulated (log_2 1.0-fold) at 40°C and repressed (log_2 1.9-fold) at 45°C. Similarly, AckA_2 (BN194_23060) was also repressed (log_2 1.2-fold) at 45°C. The protein AcyP (BN194_18620) showed no change in relative abundance at 40°C but was less abundant (log_2 1.0-fold) at 45°C.

The conversion of pyruvate to oxaloacetate involves Pyc and the reverse reaction is catalysed by PycB. While Pyc (BN194_15170) was inhibited at 40°C and 45°C, PycB (BN194_20290) was inhibited at 45°C. Oxaloacetate is an intermediate of the citrate cycle and glyoxylate metabolism, although *L. casei* has a partial TCA cycle (Morishita & Yajima 1995) and the enzymes for conversion of glyoxylate to citrate have not been identified in this species. We could not detect BN194_30060 encoding the malic enzyme and involved in the

reversible conversion of pyruvate to (S)-malate in any of the protein fractions. The non-detection level of BN194_30060 is not surprising as its expression is strictly regulated and requires the presence of malate and absence of glucose in *L. casei* BL23 (Landete *et al*. 2013). MleA (BN194_08070), involved in the conversion of the (S)-malate to L-lactate, was observed to be repressed (log_2 1.0-fold) at 40°C and suppressed beyond detection at 45°C in the CFEs. L-lactate and D-lactate can also be reversibly formed from pyruvate by Ldh and BN194_01410 (putative D-lactate dehydrogenase). Although both enzymes were not differentially modulated at 40°C, Ldh (BN194_26560) was up-regulated (log_2 1-fold) and BN194_01410 repressed (log_2 1.7-fold) at 45°C in the CFEs and highly abundant at all growth temperatures.

7.3.3 Impact of prolonged heat stress on the lipid metabolism of *L. casei* GCRL163

Proteins associated with lipid-related metabolism, identified from the *L. casei* protein fractions, were involved mostly in fatty acid, glycerolipid and glycerophospholipid metabolism. Most of the fatty acid metabolism-associated proteins showed mild to moderate upregulation at 40°C and were markedly inhibited at 45°C relative to 30°C (Figure 7. 6, Supplementary Table 7. 1 and see Supplementary Table 4.4). In the LS fraction, all of the fatty acid synthesis proteins were at markedly higher abundance at 35 and 40°C relative to 30°C (log_2 1.9-5.3-fold increases) but only AcpP_2 (BN194_22590) was seen as up-regulated in all the growth temperatures in this fraction, although this protein was seen as repressed at 45°C in the CFE and TS fractions. These proteins AccA and AccD also have a ClpP/crotonase-like domain (Hamed *et al*. 2008). Acetyl–CoA carboxylase carboxyl carrier protein AccC (BN194_22510) and uncharacterized AccC-like protein BN194_22500 are predicted cell membrane-anchored proteins which showed enhanced expression (log_2 1.6- and 1.8-fold respectively) in the CFE and up-regulated in the LS protein fractions at 40°C.

The uncharacterized protein BN194_22500 possesses a biotin carboxylation domain (Jitrapakdee & Wallace 2003). Acetyl-CoA carboxylase biotin carboxyl carrier protein AccB (BN194_22530) was not differentially expressed at 40°C in the CFE protein fraction relative to 30°C. The proteins AccB and AccC were repressed at 45°C (log_2 2.2 and 3.3-fold respectively) while AccA was not detected at this growth temperature. AccABCD is responsible for the conversion of Acetyl-CoA to malonyl-CoA in the fatty acid biosynthesis. The subsequent step in the pathway in which malonyl-CoA is acylated, carried out by malonyl CoA-acyl carrier protein transacylase FabD (BN194_22570), was not differentially expressed at 40°C but was strongly repressed (log_2 5.4-fold) at 45°C in the CFE protein fraction. Other members of the Fab family proteins that were up-regulated at 40°C included, FabG (BN194_22560) and both FabZ isoforms (FabZ (BN194_22520) and FabZ_2 (BN194_22620), approximately log_2 1.2-fold). Others including FabD, FabF (BN194_22540) and FabK (BN194_22580), were not differentially expressed while FabH (BN194_22600) was down-regulated (log_2 1.1-fold) at 40°C in the CFE protein fraction. FabF, FabG, FabH, FabK and the two FabZ isoforms were down-regulated (1.7-fold, 2.4-fold, 2.1-fold, 3.5-fold, 4.9-fold and 5.3-fold, respectively) at 45°C in the CFEs. Also, acyl-ACP thioesterase BN194_23910, involved in the late stage of fatty acid biosynthesis, showed no change in relative abundance at 40°C but was also repressed at 45°C (log_2 2.4-fold) in the CFE protein fraction. These findings demonstrated that fatty acid biosynthesis was suppressed at supra-optimal growth temperature relative to temperatures around optimum. The two exceptions were detecting two AcpP proteins in LS fractions as up-regulated at 45°C. The data also infer that many of the fatty acid synthesis proteins function at or near the cell surface.

In the glycerophospholipid metabolism, glycerol-3-phosphate dehydrogenase GpsA (BN194_10350), involved in the reversible conversion of glycerone phosphate to *sn*-glycerol-

3P, was not differentially modulated during prolonged heat stress at 40°C and 45°C in the CFEs (Figure 7. 6, Supplementary Table 7. 1). However, overexpression of PlsX (BN194_17940) , associated with the conversion of *sn*-glycerol-3-phosphate to 1-acyl-*sn*-glycerol-3P with PlsY, was observed while PlsC (BN194_17710), involved in the conversion of 1-acyl-*sn*-glycerol-3P to 1, 2 diacyl-*sn*-glycerol-3P, showed no change in relative abundance in the CFE and LS protein fractions at 40°C and 45°C. Other proteins with no differential modulation at 40°C and 45°C include BN194_11980 and BN194_09040. PlsY, CdsA, PgsA and YqlK, also involved in the metabolic pathway in *L. casei*, were not detected in all the protein fractions. DhaK (BN194_04980) and DhaM (BN194_04960) were not differentially expressed at 40°C in the CFE: DhaK was up-regulated (log_2 1.3-fold), DhaM was repressed (log_2 1.3-fold) at 45°C in the CFE protein fraction. However, these proteins, together with putative DhaL (BN194_04970, co-located with DhaK and DhaM in the genome) were all more highly abundant at 45°C (log_2 1.1-3.2-fold increases) and DhaM at 40°C in the LS fraction. Exported glycerolphosphate lipoteichoic acid synthetase LtaS1 (BN194_09110) showed enhanced expression (log_2 2.3-fold) at 40°C with no change in relative abundance at 45°C in the CFE protein fraction. This predicted integral membrane protein LtaS1 was repressed at the cell surface LS protein fraction at 45°C (see Supplementary Table 4.4). These proteins are connected to the biosynthesis of the cell wall-associated lipoteichoic acids. Putative FakA-like protein BN194_17960 (fatty acid kinase, DAK2 domain) and FakB-like proteins (such as DegV-domain, lipid-binding proteins BN194_16330, BN194_15830, and BN194_12060) were detected in CFE and LS fractions, with all of the DegV proteins up-regulated in the LS fraction at 45°C and only FakA detected in TS at 45°C but not up-regulated in the other fractions. In *S. aureus*, FakB binds to free fatty acids and presents them to FakA for phosphorylation prior to the incorporation of fatty

acids into phospholipids (a role similar to PlsX), including fatty acids scavenged from the extracellular medium and the cell membrane (Parsons *et al*. 2014).

Other proteins associated with phospholipid metabolism were detected, including lipases/esterases such as BN194_18770 (up-regulated in the LS at 45°C) and BN194_30390 (down-regulated in the CFEs at 40°C and 45°C). Putative lipid-exporting proteins, including BN194_13430 and BN194_13440, were over-expressed in the LS at the elevated growth temperature while LplJ, involved in extracellular protein lipoylation, was repressed. Glycero-lipid and glycero-phospholipid metabolism is linked to the glycolytic pathway in the strain through a reversible reaction of glycerone phosphate to glyceraldehyde-3P catalysed by the up-regulated TpiA.

7.3.4 Effect of prolonged heat stress on nucleotide and amino acid metabolism

Most of the proteins detected in *L. casei* GCRL163, which were associated with pyrimidine metabolism in the nucleotide metabolic processes, showed little in the way of differential expression at 40°C relative to 30°C in the CFE protein fraction. However, the majority of these proteins were greatly less abundant at the elevated temperature of 45°C. Detected proteins associated with purine metabolism were notably repressed at 40°C and 45°C. In the pyrimidine metabolism, PyrB, PyrC, PyrD, PyrDA, PyrF, PyrG and PyrH, were not differentially modulated at 40°C (Figure 7. 7). The proteins PyrB and PyrD become less abundant ($\log_2$ 3.4 and 2.0-fold respectively) while PyrG and PyrH were enhanced ($\log_2$ 1 and 1.9-fold respectively) at 45°C in the CFE. We detected an uncharacterized protein BN194_28370, putatively involved in the formation of dihydroorotate from N-carbamoyl-L-aspartate, to be repressed at the 40°C growth temperature. This reaction can alternatively be catalyzed by PyrC. Other proteins with inhibited expression at 40°C include the UshA

(BN194_15530), Cmk (BN194_15710) and DeoD (BN194_02810) and at 45°C include UshA (log_2 1.5-fold), Cmk (log_2 2.4-fold) and NrdE2 (log_2 1-fold). However, Dut, (BN194_24640) involved in the reversible conversion of deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP), was up-regulated at 40°C (log_2 1-fold) and 45°C (log_2 1.7-fold). The DNA-directed RNA polymerases RpoA, RpoB, RpoC and RpoZ, involved in the conversion of uridine-5'-triphosphate (UTP) and cytidine triphosphate (CTP) to RNA in the pyrimidine metabolism, were not differentially expressed at 40°C. Of these, RpoA (BN194_25960) and RpoE (BN194_26810) were enhanced while RpoZ (BN194_18100) was repressed at 45°C in the CFEs (see Chapter 6). In purine metabolism, PurB, PurC, PurD, PurK, PurK2 and PurH were less abundant (between log_2 1.7 and 10-fold) at 40°C and 45°C (Figure 7. 7). The proteins PurE, PurL, PurM, PurQ and YexA were detected only at the 30°C control growth temperature in the CFE protein fraction, which also suggests repression at 40°C and 45°C. PurA was only inhibited (log_2 1.7-fold) at 40°C while other proteins, including DeoB, Prs1 and Prs1_2, were repressed at 40°C and 45°C. These results suggest markedly repression of the purine metabolism occurs during heat stress adaptation. Several proteins involved in purine salvaging and nucleotide interconversion were detected but abundances not changed at 45°C (Ndk, GuaB, GuaC, GuaD, Xpt, Apt, Upp, DeoD, and NrdF): many of these were present at very high abundance. While nucleotide reductase NrdE_2 (BN194_16600) was down-regulated at 45°C, NrdD (anaerobic nucleotide reductase) and NrdH were both highest at 35°C and lower or not detected at the other temperatures, consistent with producing deoxynucleotides during growth at close to optimal temperature and reducing this under stress at 45°C.

In addition to higher abundance of Dut at elevated temperatures, RihC (non-specific nucleoside hydrolase), GuaA (BN194_21070) and Adk (adenylate kinase), together with

several nucleotidases, were up-regulated, indicating greater nucleotide turnover and possible degradation. However, degradation of purine bases in *L. casei*, or LAB more broadly, is not well documented despite the considerable literature on nucleotide salvaging and interconversion. In most organisms, degradation occurs via xanthine oxidase with the formation of urate and the subsequent conversion of urate into metabolites including glyoxylate, urea and ammonia. Glyoxylate is then converted by a number of routes to metabolites including formate, malate and D-glycerate, which can then enter the glycolytic pathway by conversion into 2- or 3-phospho-D-glycerate (phosphoglycerate kinase, pgk, which was up-regulated at 45°C in LS and CFE fractions). The conversion of glyoxylate to D-glycerate involves transamination reactions to produce hydroxypyruvate and the subsequent conversion of this to D-glycerate either directly or via tartronate semialdehyde (2-hydroxy-3-oxopropionate). Examining both the annotated genome of *L. casei* GCRL163 (Nahar *et al*. 2017) and the proteomic data, two proteins potentially involved in these reactions were identified: BN194_01890, hydroxypyruvate reductase, and BN194_02520, annotated as 2-hydroxy-3-oxopropionate reductase, both up-regulated at 45°C. Several other uncharacterized proteins had domains identifying them as oxido-reductases, which were up-regulated at elevated temperatures, but their functions are not known. Aminotransferases such IlvE (BN194_21620) (up-regulated at 45°C in the LS) possibly catalyse reactions involving glyoxylate and serine/alanine or glutamate, yielding hydroxypyruvate or 2-oxoglutarate and glycine in the glyoxylate pathway (Figure 7. 8). Decarboxylation of glyoxylate may potentially occur by Pox5, catalysis as reported in *L. plantarum* (Turujman & Durr 1975), resulting in formate synthesis. A model for purine degradation was developed to form a framework for future experimental research to determine the pathway/s involved in purine degradation (Figure 7. 9).

Furthermore, we detected GlnA (BN194_18340), associated with the synthesis of L-glutamine from L-glutamate, to be up-regulated (log_2 2.6-fold) at 45°C with no differential expression at 40°C in the CFEs. The reversible production of ammonium ion and alphaketoglutarate from L-glutamate is catalyzed by Gdh (Figure 7. 10, Figure 7. 11 and Supplementary Table 7. 1), which was repressed (log_2 1.6-fold) at 40°C and (log_2 2.4-fold) at 45°C. In aspartate metabolism, enzyme AsnB (BN194_22990), involved in the reversible conversion of L-aspartate to L-asparagine became less abundant. An uncharacterized protein BN194_02170 (putative aspartate racemase) generates D-aspartate from L-aspartate. This protein BN194_02170 was detected to be repressed (log_2 2.4-fold) at 45°C while AspA was not detected. We could not detect ArgG and ArgH, involved in arginine metabolism. In the lysine metabolism, DapE (BN194_13710) and DapE_2 (BN194_19740) were inhibited at 40°C and 45°C while DapF (BN194_01050) was only repressed at 45°C (see Figure 7. 8). The protein DapF was over-expressed by log_2 1.0-fold at 40°C. The conversion of L-4-aspartylphosphate to L-aspartate-4-semialdehyde is catalyzed by Asd (BN194_01060), which became over-expressed at 40°C and 45°C during heat stress. The protein Hom, involved in the reversible conversion of L-aspartate-4-semialdehyde to homoserine, was only up-regulated at 45°C. GlyA (BN194_13540), associated with the reversible reaction between serine and glycine, was not differentially modulated during prolonged heat stress in the CFEs.

7.4 Discussion

Lactic acid bacteria can adapt under different environmental stress conditions by modulating the synthesis of proteins and metabolites, which impact on their metabolic processes and energy requirements (Papadimitriou *et al*. 2016). Metabolic pathways for substrate catabolism are often rerouted under stress conditions to generate additional energy and cushion the impact of harsh conditions (De Angelis *et al*. 2004). The current study

revealed more findings on the impact of prolonged heat stress on the modulation of proteins associated with energy metabolism and other metabolic processes in *L. casei* GCRL163. Our proteomic analysis suggests that the sugars used by *L. casei* GCRL163 under prolonged heat stress as carbon sources, at the mid-exponential growth phase and growth temperature close to the optimum, are not entirely sourced from the MRS growth medium but come from endogenous nucleotide sugar derivatives, probably during exopolysaccharide and peptidoglycan degradation and turnover by the cell wall hydrolases. In a previous finding, we reported increased synthesis of several cell wall hydrolases during prolonged heat stress at the growth temperature of 40°C and some of these proteins were less abundant at the elevated growth temperature of 45°C relative to 30°C and some with high relative abundances in the extracellular culture fluid (Chapter 4). Cell wall hydrolases are involved in cell wall remodelling and turnover, contributing to cell division and bacterial growth (Regulski *et al*. 2012). Like most LAB, *L. casei* synthesizes exopolysaccharides, as components of the cell envelope, which are constituted by repeating units of D-galactose, D-glucose, and D-rhamnose and their amino-and acetylated derivatives (De Vuyst & Degeest 1999; Mozzi *et al*. 2001). As a result of the turnover activities of the cell wall hydrolases, endogenous sugar derivatives could be generated by *L. casei* GCRL163 under prolonged heat stress. Intriguingly, in the current study, proteins associated with the uptake of β-glucoside, mannose, fructose, and galactose in the PEP-PTS, were over-expressed by heat stress (Figure 7. 1). Bacterial cells, cultivated in a glucose-rich medium under non-stressed conditions, are known to activate glucose uptake by PEP-PTS while inhibiting the transport of other sugars (Papadimitriou *et al*. 2016). The PtsG-1 (BN194_28320), involved in PTS glucose uptake, was not abundantly expressed despite the MRS broth used for the culturing strain *L. casei* GCRL163 was supplemented with an additional 1% glucose. The main PTS glucose transporter in *L. casei* is the mannose-class transporter ManABCD (Veyrat *et al*. 1994). Apart

from ManD up-regulated at 45°C, expression of this cluster of PTS transporters was repressed or not detected at 45°C in the CFEs and LS fraction, suggesting a minimal glucose uptake at the elevated growth temperature of 45°C. While other PTS proteins which are known to transport multiple sugars may be involved in glucose uptake (Monedero *et al*. *et al*. 2007), the observed upregulation of proteins involved in the uptake of sugars other than glucose such as mannose, fructose and galactose suggested a preference for uptake of endogenously-derived sugars at temperatures close to but higher than the optimum. This is understandable as these sugars can be phosphorylated and used as substrates by *Lactobacillus* spp (Pessione *et al*. 2010). The phosphorylated substrates can directly enter into the glycolytic pathway and be used to generate energy, thereby generating a net increase in ATP (Cohen *et al*. 2006). Additionally, we identified a fructose-specific enzyme IIABC complex (FruA3) which catalyzes the synthesis of fructose-1-phosphate in the PTS phosphorylation reaction. This enzyme was highly up-regulated during prolonged heat stress. This suggests the involvement of the fructose PTS in heat stress response in *L. casei* GCRL163. Induction of the phosphoenolpyruvate-fructose phosphotransferase system in the presence of fructose has been reported in *L. monocytogenes* (Mitchell *et al*. 1993) and *L. brevis* (Saier *et al*. 1996). The results suggest that growth temperature and prolonged heat stress can markedly influence carbohydrate uptake in LAB.

Our proteomic data further revealed the preference in sugar utilization by *L. casei* GCRL163 during prolonged heat stress. Although the synthesis of proteins associated with the uptake of carbohydrates other than glucose was up-regulated by thermal stress, proteins associated with the catabolism of these carbohydrates, including galactose and fructose were either down-regulated or not differentially expressed. Notably, GalE_2, GalT, BGAL17, Cap4C and BN194_07390 involved in the galactose and sucrose metabolism were repressed

at 45°C. These findings coincide with the up-regulated synthesis of several enzymes in the glycolytic pathway involved in the glucose metabolism, including PfkA associated with the formation of fructose-1,6P2 from fructose-6P. Moreover, glycolysis is an amphibolic system where high-energy ATP is catabolically generated and the precursors for cellular components are synthesized using the energy of ATP (Wolfe 2015). In the glycolytic pathways, several proteins including the GlcK, PfkA, Fba_2, Gap, Gap_2, Pgk, GpmA2 and Eno were over-expressed only at the elevated temperature of 45°C, mostly in the CFE fractions (Figure 7. 2). This result suggests an attempt to increase energy generation in form of ATP. The generation of more energy is an adaptive mechanism to cope with greater levels of energy-dependent protein folding and turnover that characterizes heat stress. In keeping up with the high-energy demand at the elevated temperature, *L. casei* GCRL163 activated metabolic networks that could channel biosynthetic precursors into the glycolytic pathways to enhance energy production. Notably, NagA and ManD, involved in the generation of GlcN-6P from GlcNAc-6P in the nucleotide sugar metabolism, were moderately up-regulated and NagB, which converted the GlcN-6P to fructose-6P, was also enhanced at the elevated growth temperature (Figure 7. 3). Furthermore, LacC, LacD2, LacD2_2 and PfkA, involved in the synthesis of D-glyceraldehyde-3P through the tagatose metabolism, were up-regulated at the elevated growth temperature (Figure 7. 3). Elevated growth temperature also induced overexpression of GntK and Gnd with an inhibition of RpiA_2, RbsK and Prs1 in the Entner-doudoroff pathway (Figure 7. 2). This suggests shunting of the Entner-doudoroff pathway into the glycolytic pathways for the synthesis of fructose-6P instead of generating PRPP which is a substrate for purine, pyrimidine and histidine metabolism. Moreover, phosphoketolase XpkA, involved in the generation of glyceraldehyde-3P from xylulose-5P, was up-regulated. Glycerone phosphate from the glycerophospholipid catabolism could also be channelled into the synthesis of glyceraldehyde-3P, as TpiA was over-expressed by heat stress. Moreover,

DhaM, putative DhaL and DhaK, converting glycerone to the glycerone phosphate, were over-expressed, further suggesting that *L. casei* was channeling carbon source into glycolysis and phospholipid synthesis from phospholipid metabolism. The alternative carbon sources are channeled mainly into glycolysis via fructose-6P and glyceraldehyde-3P.

Furthermore, *Lactobacillus* spp. can modify pyruvate metabolism to modulate the synthesis of proteins associated with the generation of energy-rich compounds such as ATP and NADH, exopolysaccharides and metabolic end-products and intermediates such as lactate, acetate, malate, oxaloacetate, succinate, acetyl-CoA and acetaldehyde under different stress conditions (Angelis *et al*. 2016). In this study, Ldh was up-regulated at the elevated growth temperature. Ldh, involved in the reversible conversion of pyruvate to L-lactate, has been associated with quorum sensing mechanisms in *L. plantarum* (Di Cagno *et al*. 2009) and enhanced tolerance to heat stress in *L. gasseri* (Suokko *et al*. 2008). Ldh is also involved in regenerating oxidized cofactors, which can be used in other metabolic pathways (Zhu *et al*. 2015). Although proteins associated with the formation of acetate from pyruvate were repressed at elevated growth temperature of 45, expression of several ATP synthase proteins was enhanced, suggesting generation of energy from alternative sources. Acetyl-CoA is channeled into fatty acid metabolism and provides building substrates for the synthesis of essential molecules, including amino acids, nucleotides and exopolysaccharides (Wolfe 2015). As a response to prolonged heat stress, several enzymes involved in the generation of fatty acids from the acetyl-CoA in the fatty acid biosynthesis pathways were moderately up-regulated including AccA, AccC, FabG and FabZ at 40°C while all the proteins in the pathways were markedly repressed at the elevated growth temperature of 45°C (Figure 7. 6). Fatty acid biosynthesis is an energy intensive process, involving in cell membrane fluidity (Jerga & Rock 2009; Louesdon *et al*. 2015) and protection against environmental stress

conditions (Rivas-Sendra *et al*. 2011). Furthermore, abundant expression of lipases, including MqhD, at elevated growth temperature suggested phospholipid degradation as evidenced by modification of fatty acids (Chapter 3), indicating cell memebrane reshaping under prolonged heat stress.

The pattern of modulation of the enzymes involved in the synthesis of peptidoglycan suggests reduced peptidoglycan synthesis at an elevated growth temperature of 45°C. This is evidenced by Pmi, GlmU, GlmS, MurA2 and MurB becoming less abundant (Figures 7.3 and 7.4). The reduced peptidoglycan/teichoid acid synthesis could also be responsible for cell fragility and increased cell lysis at 45°C (Chapter 4). Although, the inhibition could pose considerable challenges for the maintenance of the cell envelope at the extreme growth temperature, it might be part of the adaptive mechanisms.

Nucleotides play essential roles in RNA and DNA synthesis, activation of precursors in polysaccharide and lipid synthesis, energy donor for cellular processes, constituents of coenzymes and regulation of cellular processes (Kilstrup *et al*. 2005). Nucleotides can either be acquired by lactobacilli through de novo synthesis or uptake from the growth medium containing purine or pyrimidines (Angelis *et al*. 2016). PRPP is a precursor for purine and pyrimidine metabolism and thus plays a role in nucleobase salvage, biosynthesis of NAD^+ and $NADP^+$ coenzymes and may acts as the inducer in PurR-mediated gene regulation (Kilstrup *et al*. 2005). In this study, prolonged heat stress resulted in inhibited synthesis of Prs1 and Prs1_2, involved in the PRPP synthesis (Figure 7. 7). Almost all the enzymes involved in purine biosynthesis were inhibited including proteins in the *pur* operon. The Pur proteins are only expressed when sufficient PRPP is available, assuming the system operates as found in *B. subtilis* (Ebbole & Zalkin 1989). The *pur* operon repressor (PurR_2) was

repressed by prolonged thermal stress as demonstrated in our previous proteomic findings (Chapter 6), in contrast to expectation. The PurR regulon was found to be induced under purine starvation in *Lactococcus lactis* (Beyer *et al*. 2003). Furthermore, several proteins involved in pyrimidine metabolism were repressed by heat stress. In amino acid metabolism, Asd and Hom involved in the synthesis of homoserine, GlnA converting L-glutamate to L-glutamine was over-expressed while proteins involved in L-asparagine metabolism were inhibited. In response to prolonged heat stress, we have previously reported inhibited synthesis of several proteins involved in the tRNA aminoacyl synthesis, ribosomal proteins, protein translation, transcription-associated proteins, although some proteins in the cellular processes were up-regulated enough to maintain growth and adaptation to the heat stress (Chapter 3). These findings suggest repressed biosynthesis of proteins in *L. casei* GCRL163 during the prolonged thermal stress.

This study has provided new insights into how *L. casei* GCRL163 manages metabolic processes to adapt to prolonged heat stress. Several peptidases were up-regulated with enhanced expression of proteins associated with amino acid uptake, also suggesting scanvenging rather than production of amino acids during prolonged heat stress. The overall rerouting of metabolic pathways by *L. casei* GCRL163, during prolonged heat stress at mid-exponential growth phase, appears to revolve around harnessing sufficient metabolic energy to sustain cellular and molecular processes required for cell survival. Additional cellular carbon sources are scavenged from different metabolic pathways and shunted into the glycolytic pathway to generate more energy. High energy-requiring biosynthetic pathways, such as fatty acid biosynthesis, were repressed while adenylate kinase, involved in energy homeostasis, was up-regulated in the cell surface TS fraction. These mechanisms constitute part of PHSR of *L. casei* GCRL163 to prolonged thermal stress.

7.5 Figures

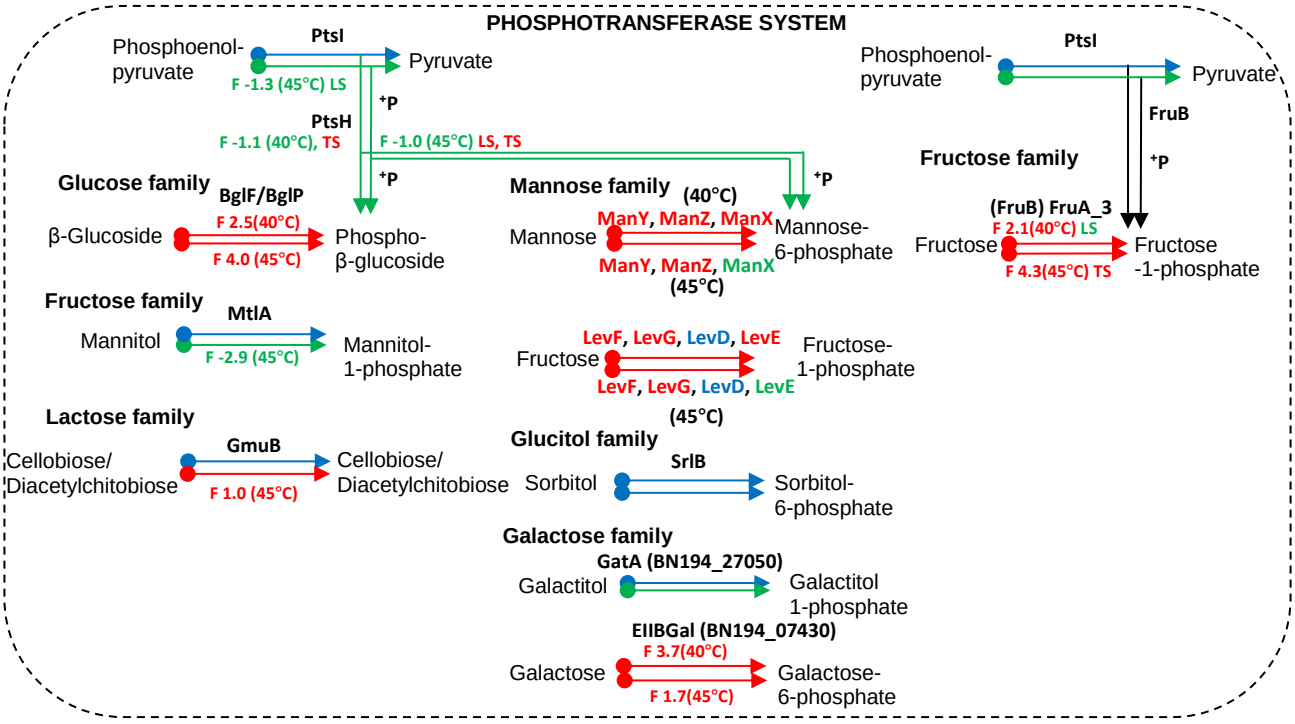


Figure 7. 1. Schematic representation of the identified proteins in *L. casei* GCRL163 involved in the phosphoenolpyruvate (PEP)-dependent carbohydrate-transport phosphotransferase system (PTS) under prolonged heat stress. All the metabolic maps in the current study were adapted from KEGG pathways (Kanehisa & Goto 2000) and the construction of the maps was based on two-sample-t-test difference of log_2-transformed label-free quantitative proteomic data from cells grown under controlled cultured conditions at 40°C and 45°C relative to 30°C. Where applicable, the coloured arrows represent up- (red) or downregulation (green) or not differentially modulated (blue) or not detected (purple) or regulation of more than one protein with differed expression (brown) or not known to be present in *L. casei* (black) while the dashed-line arrow represents a series of reactions, where every enzyme or substrate not shown. Up-regulated proteins are indicated by red fold change (F) values or proteins in red colour and down-regulated proteins by green F-values or proteins in green colour, with respective growth temperature and fold change values indicated. PtsI, Phosphoenolpyruvate-protein phosphotransferase; PtsH, phosphocarrier protein HPr; BglP, PTS system beta-glucoside-specific EIIBCA component; MtlA, PTS system mannitol-specific EIICBA component; GmuB, PTS system (lactose/DACB/beta-glucoside family) subunit IIB; ManY, PTS (mannose/fructose/sorbose family) IIC; ManZ_9, PTS (mannose/fructose/sorbose

family) IID; ManX, PTS (mannose/fructose/sorbose family) IIAB; LevF, PTS (glucose/mannose family) IIC; LevG, PTS (glucose/mannose family) IID; LevD, PTS (glucose/mannose family) IIA; LevE, PTS (mannose/fructose/sorbose family) IIB subunit; SrlB, PTS (sorbitol/glucitol family) subunit IIA; GatA, PTS (galactitol family) IIA-2; EIIBGal, PTS (Galactitol family) IIB and FruA_3, PTS (fructose family) subunit IIABC.

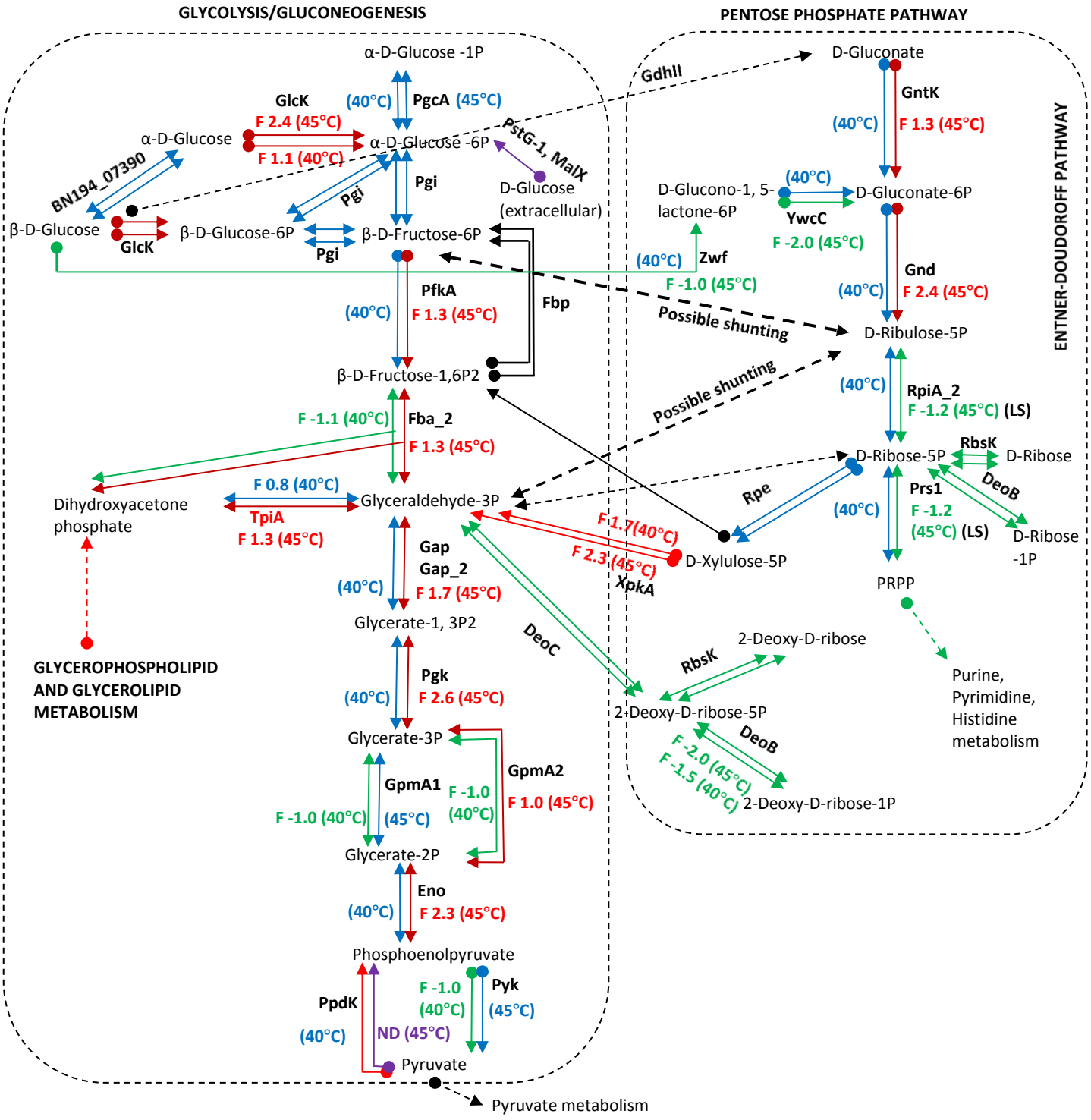


Figure 7. 2. Metabolic map of the identified proteins in *L. casei* GCRL163 involved in the glycolytic and pentose phosphate pathways under prolonged heat stress. Where applicable, protein fractions are denoted by LS (lithium chloride-sucrose), TS (trypsin shaving) and ECF (extracellular fluid) extracts. GlcK, glucokinase; PgcA, phosphoglucomutase; Pgi, glucose-6-phosphate isomerase; BN194_07390, aldose 1-epimerase; PfkA, 6-phosphofructokinase;

Fba_2, fructose-bisphosphate aldolase; Fbp, fructose-1,6-bisphosphatase 1; Gap, Gap_2, glyceraldehyde-3-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; GpmA1, GpmA2, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase; PpdK, pyruvate, phosphate dikinase; GntK, gluconokinase; Gnd, 6-phosphogluconate dehydrogenase; YwcC, 6-phosphogluconolactonase; Zwf, glucose-6-phosphate 1-dehydrogenase; RpiA_2, ribose-5-phosphate isomerase A; RbsK, ribokinase; Prs1, ribose-phosphate pyrophosphokinase 1; DeoB, phosphopentomutase; GdhII, glucose 1-dehydrogenase 2; XpkA, xylulose-5-phosphate phosphoketolase and Rpe, ribulose-phosphate 3-epimerase.

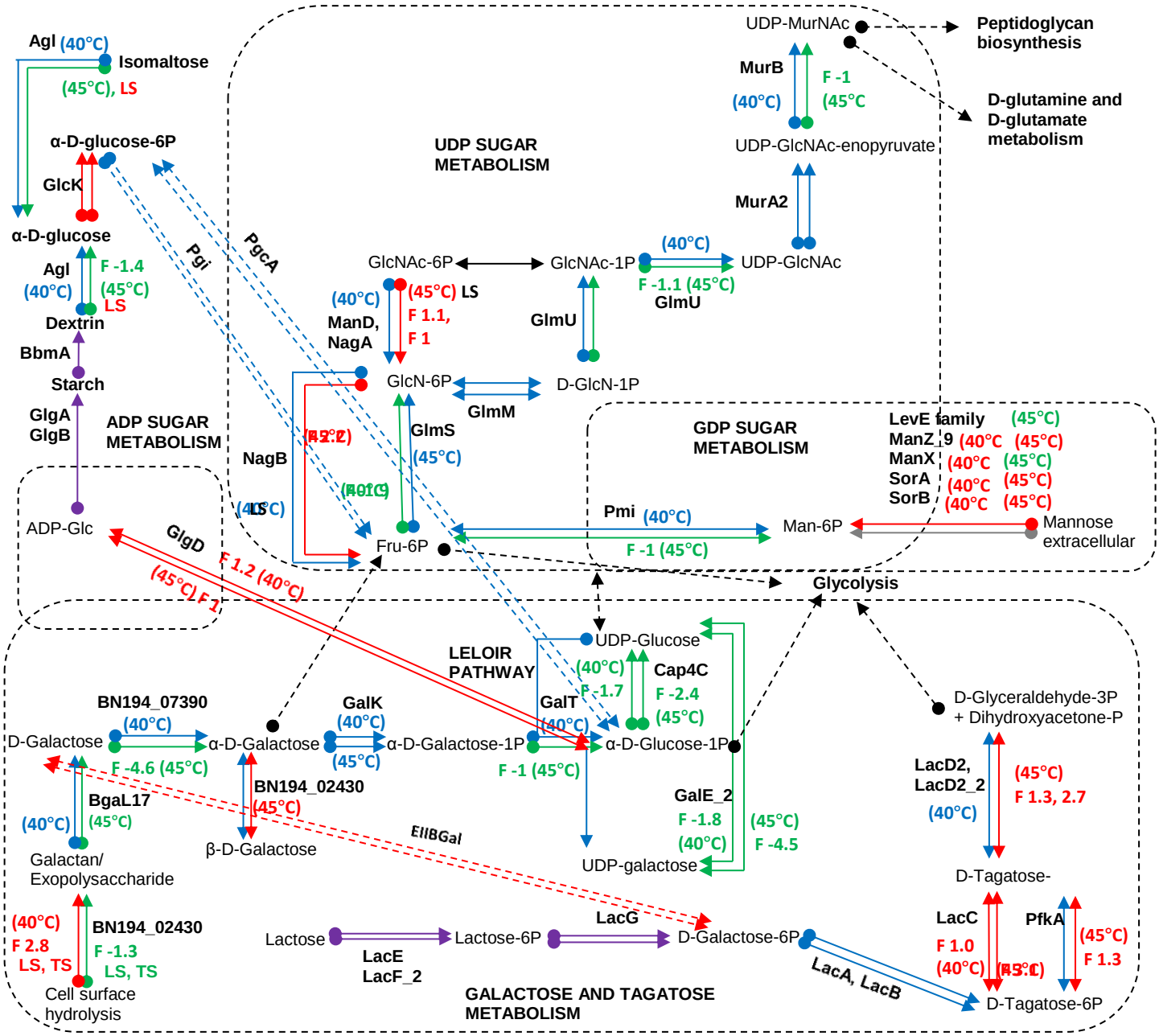


Figure 7. 3. Schematic metabolic map demonstrating change in differential abundances of proteins involved in the metabolism of galactose, tagatose and nucleotide and amino sugars in *L. casei* GCRL163 under prolonged heat stress. MurB, UDP-N-acetylenolpyruvoylglucosamine reductase; MurA, UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2; GlmM, phosphoglucosamine mutase; GlmU, glucosamine-1-phosphate N-acetyltransferase/UDP-N-acetylglucosamine pyrophosphorylase; GlmS, glucosamine-fructose-6-phosphate aminotransferase; ManD, N-acetylglucosamine-6-phosphate deacetylase; NagA, N-acetylglucosamine-6-phosphate deacetylase; NagB,

glucosamine-6-phosphate deiminase; GlgA, glycogen/starch synthase; GlgB, 1,4-alpha-glucan branching enzyme; GlgD, glucose-1-phosphate adenylyltransferase; Pmi, mannose-6-phosphate isomerase; ManX, PTS (mannose/fructose/sorbose family) IIAB; LevE, PTS (mannose/fructose/sorbose family) IIB subunit; ManZ_9, PTS (mannose/fructose/sorbose family) IID; SorA, PTS (mannose/fructose/sorbose family) IIC; SorB, PTS (mannose/fructose/sorbose family) IIB; EIIBGal, PTS (Galactitol family) IIB; BN194_07390, aldose 1-epimerase; BN194_02430, cell wall hydrolase; GalK, galactokinase; GalT, galactose-1-phosphate uridylyltransferase; GalE_2, UDP-glucose 4-epimerase; Cap4C, UTP-glucose-1-phosphate uridylyltransferase; BGAL17, beta-galactosidase; LacD2, LacD2_2, tagatose 1,6-diphosphate aldolase 2; LacC, tagatose-6-phosphate kinase; LacB, galactose-6-phosphate isomerase subunit LacB; LacA, galactose-6-phosphate isomerase subunit LacA; LacG, lactose transport system permease protein LacG; LacF, lactose transport system permease protein LacF; Agl, oligo-1,6-glucosidase; PfkA, 6-phosphofructokinase; BbmA, maltogenic amylase; and GlcK, glucokinase.

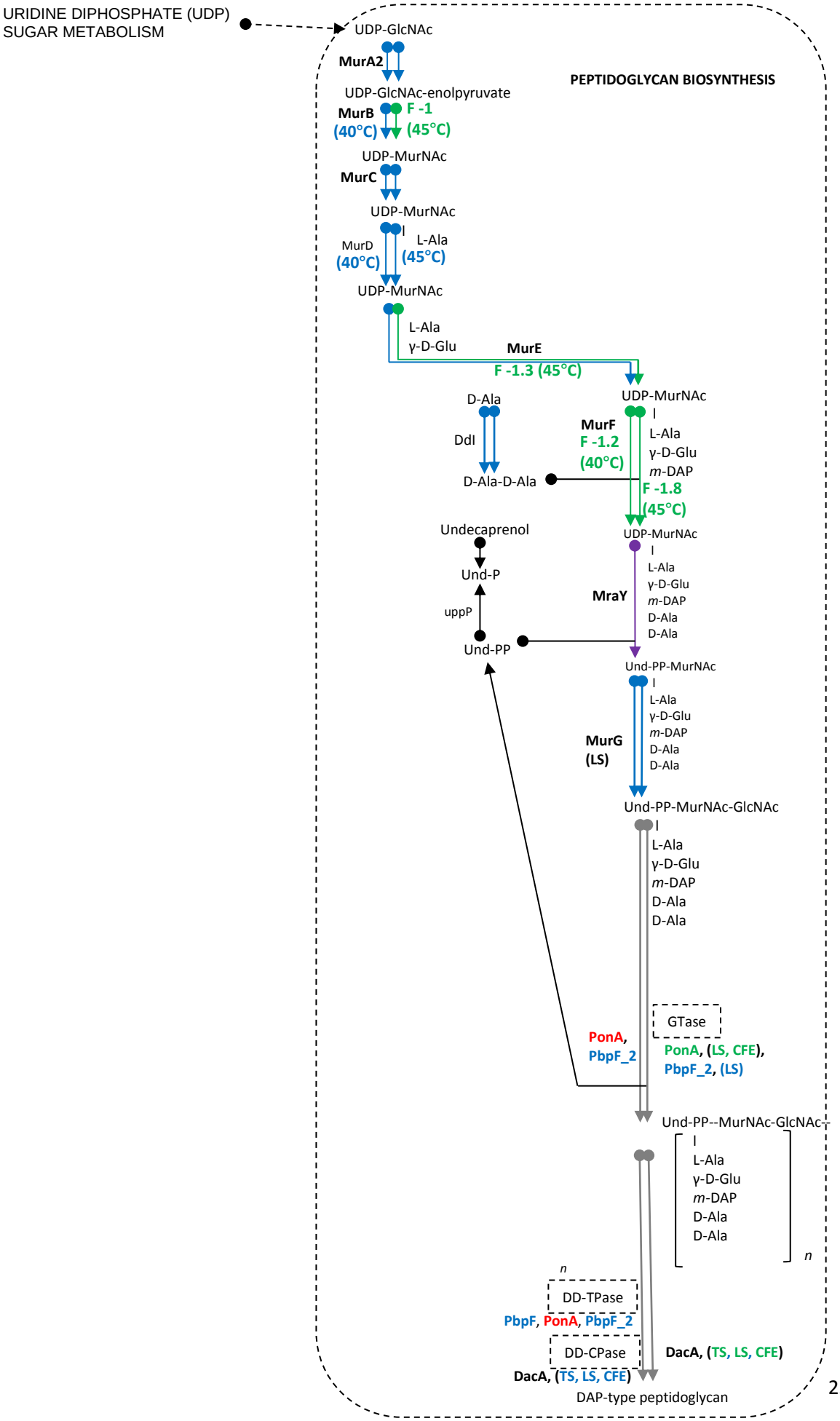

URIDINE DIPHOSPHATE (UDP)
SUGAR METABOLISM
PEPTIDOGLYCAN BIOSYNTHESIS
UDP-GlcNAc
MurA2
UDP-GlcNAc-enolpyruvate
MurB
(40°C)
F -1
(45°C)
UDP-MurNAc
MurC
UDP-MurNAc
MurD
(40°C)
L-Ala
(45°C)
UDP-MurNAc
L-Ala
γ-D-Glu
MurE
F -1.3 (45°C)
D-Ala
DdI
D-Ala-D-Ala
UDP-MurNAc
MurF
F -1.2
(40°C)
L-Ala
γ-D-Glu
m-DAP
F -1.8
(45°C)
Undecaprenol
Und-P
uppP
Und-PP
MraY
UDP-MurNAc
L-Ala
γ-D-Glu
m-DAP
D-Ala
D-Ala
Und-PP-MurNAc
L-Ala
γ-D-Glu
m-DAP
D-Ala
D-Ala
MurG
(LS)
Und-PP-MurNAc-GlcNAc
L-Ala
γ-D-Glu
m-DAP
D-Ala
D-Ala
GTase
PonA,
PbpF_2
PonA, (LS, CFE),
PbpF_2, (LS)
Und-PP--MurNAc-GlcNAc-
L-Ala
γ-D-Glu
m-DAP
D-Ala
D-Ala
n
n
DD-TPase
PbpF, PonA, PbpF_2
DD-CPase
DacA, (TS, LS, CFE)
DacA, (TS, LS, CFE)
DAP-type peptidoglycan

Figure 7. 4. Metabolic map of late peptidoglycan biosynthesis in *L. casei* GCRL163 under prolonged heat stress. MurA2, UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2; MurB, UDP-N-acetylenolpyruvoylglucosamine reductase; MurC, UDP-N-acetylmuramate-L-alanine ligase; MurD, UDP-N-acetylmuramoylalanine-D-glutamate ligase; MurE, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase; MurF, UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase; MurG, UDP-N-acetylglucosamine-N-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol N- acetylglucosamine transferase; Mray, phospho-N-acetylmuramoyl-pentapeptide-transferase; Ddl, D-alanine-D-alanine ligase; PonA, peptidoglycan glycosyltransferase; PbpF, PbpF2, peptidoglycan glycosyltransferase/transpeptidase/penicillin-binding protein 1F and DacA, D-alanyl-D-alanine carboxypeptidase dacA.

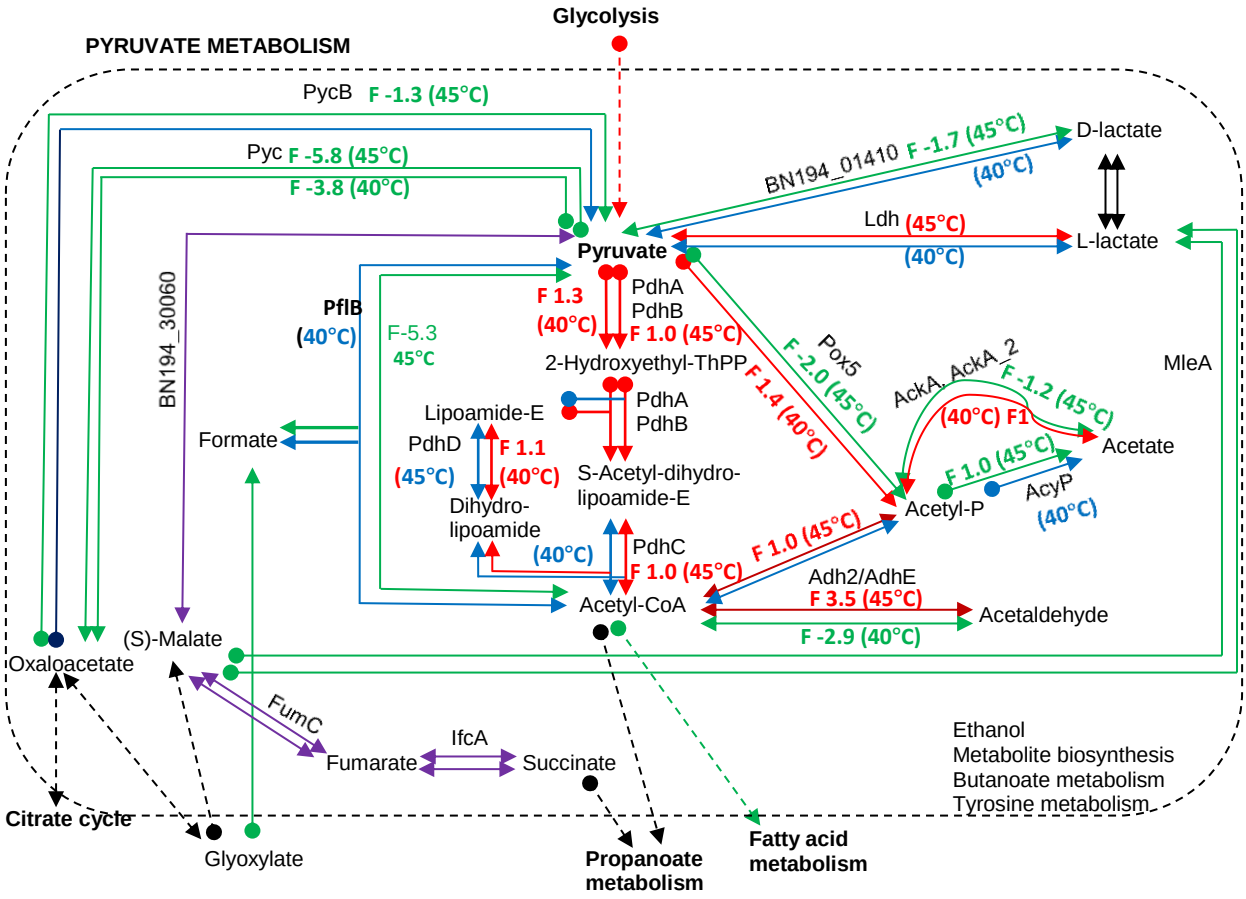


Figure 7. 5. Schematic metabolic map of the pyruvate metabolism of *L. casei* GCRL163, showing change in abundances of detected proteins under prolonged heat stress. Ldh, Lactate dehydrogenase; PyC, pyruvate carboxylase; PycB, oxaloacetate decarboxylase, alpha subunit; MleA, malate dehydrogenase; PflB, formate acetyltransferase; PdhABCD, pyruvate dehydrogenase complex; PoX5, pyruvate oxidase; AdhE, acetaldehyde-CoA dehydrogenase; Ack, Ack_2, acetate kinase; Pta, phosphate acetyltransferase; AcyP, acylphosphatase; BN194_01410, D-lactate dehydrogenase, putative; BN194_08220, HAD-like hydrolase superfamily protein phosphoglycolate phosphatase-like; BN194_30060, NAD-dependent malic enzyme; FumC, fumarate hydratase; IfcA, fumarate reductase flavoprotein subunit; GlcD, glycolate oxidase subunit GlcD; and GlnA_2, glutamine synthetase.

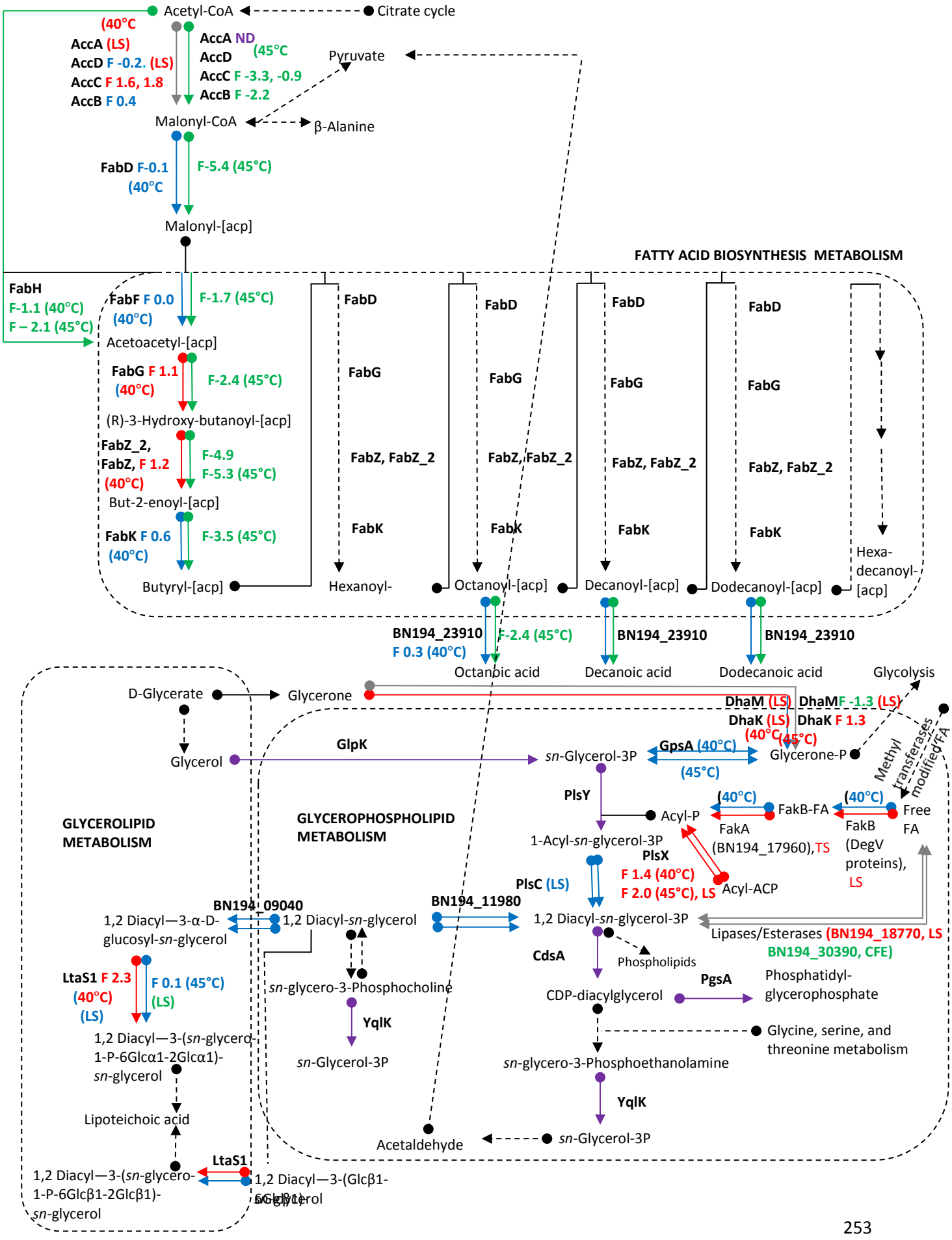

Acetyl-CoA
Citrate cycle
(40°C
AccA (LS)
AccD F -0.2. (LS)
AccC F 1.6, 1.8
AccB F 0.4
AccA ND
AccD (45°C
AccC F -3.3, -0.9
AccB F -2.2
Pyruvate
Malonyl-CoA
β-Alanine
FabD F-0.1 (40°C
F-5.4 (45°C)
Malonyl-[acp]
FATTY ACID BIOSYNTHESIS METABOLISM
FabH
F-1.1 (40°C)
F – 2.1 (45°C)
FabF F 0.0 (40°C)
F-1.7 (45°C)
Acetoacetyl-[acp]
FabG F 1.1 (40°C)
F-2.4 (45°C)
(R)-3-Hydroxy-butanoyl-[acp]
FabZ_2, FabZ, F 1.2 (40°C)
F-4.9
F-5.3 (45°C)
But-2-enoyl-[acp]
FabK F 0.6 (40°C)
F-3.5 (45°C)
Butyryl-[acp]
FabD
FabG
FabZ, FabZ_2
FabK
Hexanoyl-
Octanoyl-[acp]
Decanoyl-[acp]
Dodecanoyl-[acp]
Hexa-decanoyl-[acp]
BN194_23910
F 0.3 (40°C)
F-2.4 (45°C)
BN194_23910
BN194_23910
Octanoic acid
Decanoic acid
Dodecanoic acid
Glycolysis
D-Glycerate
Glycerone
DhaM (LS)
DhaK (LS)
DhaM F -1.3 (LS)
DhaK F 1.3 (40°C, 45°C)
Glycerol
GlpK
sn-Glycerol-3P
GpsA (40°C)
(45°C)
Glycerone-P
Methyl transferases modified FA
PlsY
(40°C)
FakB-FA
(40°C)
Free FA
Acyl-P
FakA (BN194_17960),TS
FakB (DegV proteins), LS
1-Acyl-sn-glycerol-3P
PlsX
F 1.4 (40°C)
F 2.0 (45°C), LS
Acyl-ACP
PlsC (LS)
BN194_11980
BN194_09040
1,2 Diacyl—3-α-D-glucosyl-sn-glycerol
1,2 Diacyl-sn-glycerol
1,2 Diacyl-sn-glycerol-3P
Lipases/Esterases (BN194_18770, LS
BN194_30390, CFE)
CdsA
Phospholipids
LtaS1 F 2.3 (40°C) (LS)
F 0.1 (45°C) (LS)
sn-glycero-3-Phosphocholine
PgsA
Phosphatidyl-glycerophosphate
CDP-diacylglycerol
YqlK
Glycine, serine, and threonine metabolism
1,2 Diacyl—3-(sn-glycero-1-P-6Glcα1-2Glcα1)-sn-glycerol
sn-Glycerol-3P
sn-glycero-3-Phosphoethanolamine
Lipoteichoic acid
YqlK
Acetaldehyde
sn-Glycerol-3P
LtaS1
1,2 Diacyl—3-(sn-glycero-1-P-6Glcβ1-2Glcβ1)-sn-glycerol
1,2 Diacyl—3-(Glcβ1-6Glcβ1)-sn-glycerol
GLYCEROLIPID METABOLISM
GLYCEROPHOSPHOLIPID METABOLISM

Figure 7. 6. Schematic metabolic map of the proteins in *L. casei* GCRL163 involved in the fatty acid biosynthesis and metabolism of glycerophospholipid and glycerolipid under prolonged heat stress. AccABCD, Acetyl-CoA carboxylase carboxyl transferase complex; FabDFGHK, 3-oxoacyl-ACP synthase complex; GpsA, glycerol-3-phosphate dehydrogenase; DhaMK, dihydroxyacetone kinase complex; PlsC, 1-acyl-sn-glycerol-3-phosphate acyltransferase; PlsX, glycerol 3-phosphate acyltransferase; PlsY, glycerol-3-phosphate O-acyltransferase; BN194_23910, acyl-ACP thioesterase; BN194_11980, diacylglycerol kinase; BN194_09040, 1,2-diacylglycerol 3-glucosyltransferase; LtaS1, exported glycerolphosphate lipoteichoic acid synthetase; CdsA, phosphatidate cytidylyltransferase; PgsA, CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyl-transferase; YqiK, putative inner membrane protein YqiK; FakA, fatty acid kinase-ATP binding-domain (BN194_17960); FakB, kinase-fatty acid-binding protein (DegV proteins - BN194_16330, BN194_15830, and BN194_12060), and Lipases/Esterases (BN194_30390, BN194_18770).

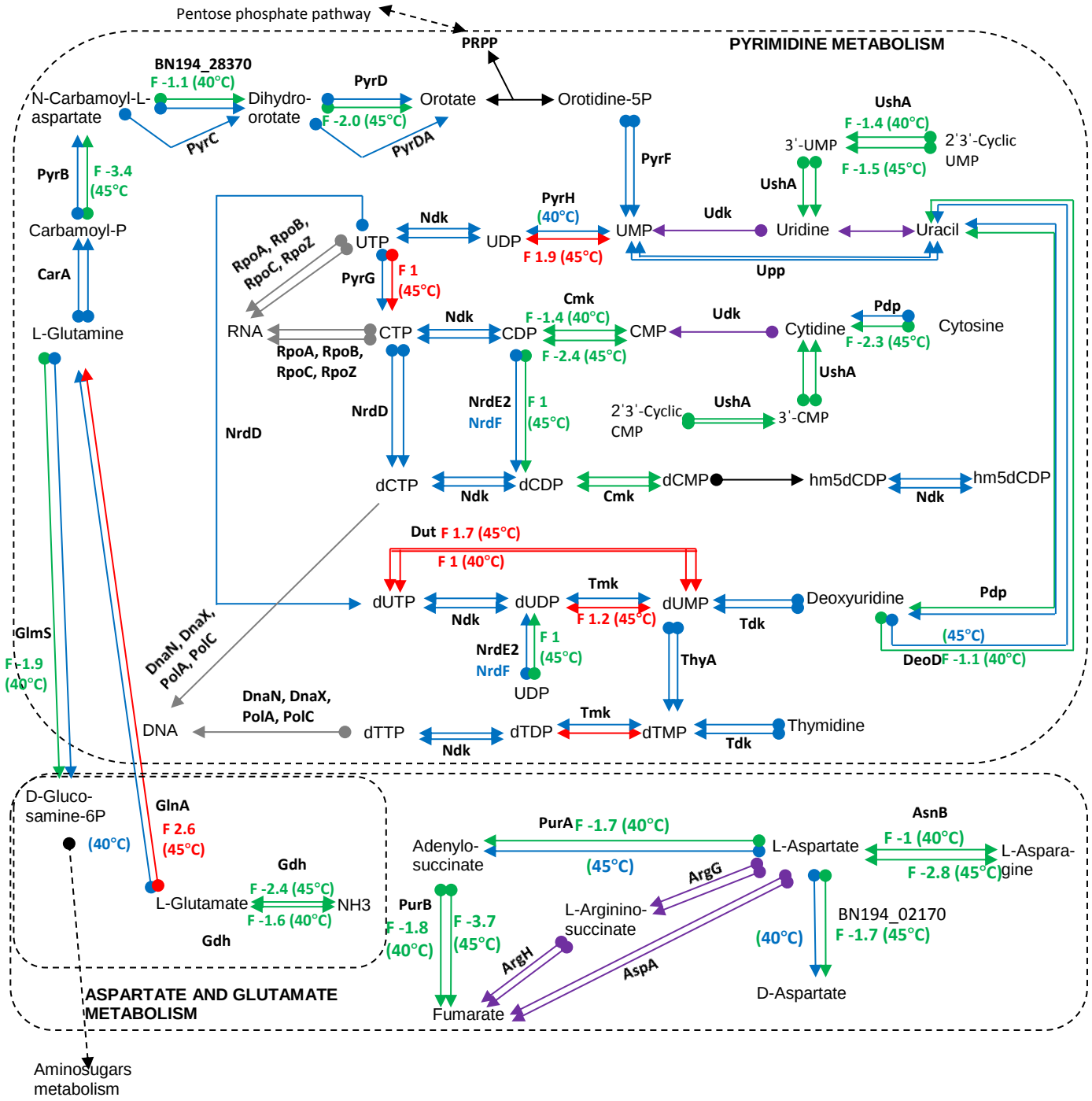


Figure 7. 7. Schematic representation of the metabolism of the nucleotides and amino acids (glutamate and aspartate) in *L. casei* GCRL163 under prolonged heat stress. Proteins detected include: CarA; carbamoyl-phosphate synthase small subunit; PryB, aspartate carbamoyltransferase; PyrC, dihydroorotase; PyrD, dihydroorotate dehydrogenase A (fumarate); PyrDA, dihydroorotate dehydrogenase A; PyrH, uridylate kinase; PyrF, orotidine 5'-phosphate decarboxylase; PyrG, CTP synthase; BN194_28370, metallo-dependent hydrolase; UshA, 5-nucleotidase 2,3-cyclic-nucleotide 2-phosphodiesterase; Upp, uracil

phosphoribosyltransferase; Pdp, pyrimidine-nucleoside phosphorylase; Cmk, cytidylate kinase; Ndk, nucleoside diphosphate kinase; RpoABCZ, DNA-directed RNA polymerase complex; NrdD, anaerobic ribonucleoside-triphosphate reductase; Tmk, thymidylate kinase; DeoD, purine nucleoside phosphorylase; ThyA, thymidylate synthase; NrdF, ribonucleoside-diphosphate reductase subunit beta; NrdE2, ribonucleoside-diphosphate reductase subunit alpha 2; Tdk, thymidine kinase/deoxyuridine kinase; GlmS, glucosamine--fructose-6-phosphate aminotransferase; GlnA, glutamine synthetase; Gdh, NADP-specific glutamate dehydrogenase; PurA, adenylosuccinate synthetase; PurB, adenylosuccinate lyase; PurC, phosphoribosylaminoimidazole-succinocarboxamide synthase; PurD, phosphoribosylamine-glycine ligase; PurE, N5-carboxyaminoimidazole ribonucleotide mutase; PurH, phosphoribosylaminoimidazole carboxamide formyltransferase/inosine-monophosphate cyclohydrolase; PurLMNQ, phosphoribosylformylglycinamidine synthase complex; AsnB, asparagine synthetase (glutamine-hydrolyzing) 1; ArgG, BN194_02170, aspartate racemase; DeoB, phosphopentomutase; PgcA, phosphoglucomutase; Prs1, ribose-phosphate pyrophosphokinase 1; Apt, adenine phosphoribosyltransferase. Abbreviations used include: UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; CMP, cytidine monophosphate; CDP, cytidine diphosphate; CTP, cytidine triphosphate; TMP, thymidine monophosphate; TDP, thymidine diphosphate; TTP, thymidine triphosphate; PRPP, phosphoribosyl pyrophosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, aminoimidazole ribonucleotide; CAIR, phosphoribosyl carboxyaminoimidazole; SAICAR, succinocarboxyamide carboxyaminoimidazole ribonucleotide; AICAR, aminoimidazole carboxamide ribonucleotide; FAICAR, formaminoimidazole carboxamide ribonucleotide; IMP, inosine monophosphate; AMP, adenosine monophosphate.

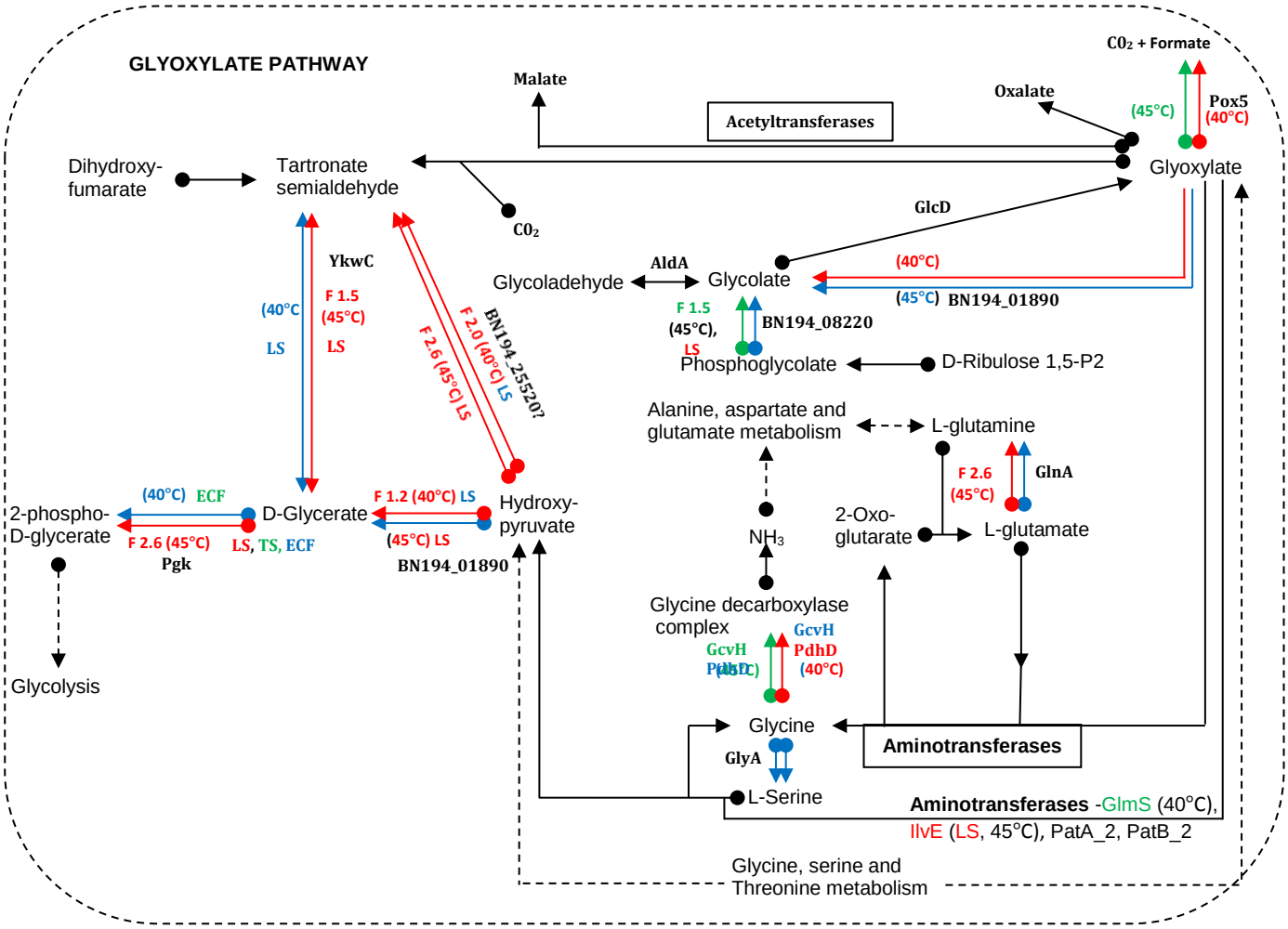


Figure 7. 8. Proposed metabolic map of the glyoxylate pathway in *L. casei* GCRL163, illustrating differential expression of proteins identified under prolonged heat stress. Pgk, phosphoglycerate kinase; YkwC, 3-hydroxyisobutyrate dehydrogenase family protein; BN194_01890, D-glycerate dehydrogenase/hydroxypyruvate reductase family protein; BN194_25520, NAD (P)H-dependent dehydrogenase/reductase; GlyA, serine hydroxymethyltransferase; GcvH, glycine cleavage system H protein; PdhD, dihydrolipoyl dehydrogenase; GlnA, glutamine synthetase; BN194_08220, HAD-like hydrolase superfamily protein Gph-like; GlmS, glucosamine-fructose-6-phosphate aminotransferase; IlvE, branched-chain amino acid aminotransferase, PatA_2, aromatic amino acid aminotransferase; and PatB_2, cystathionine beta-lyase.

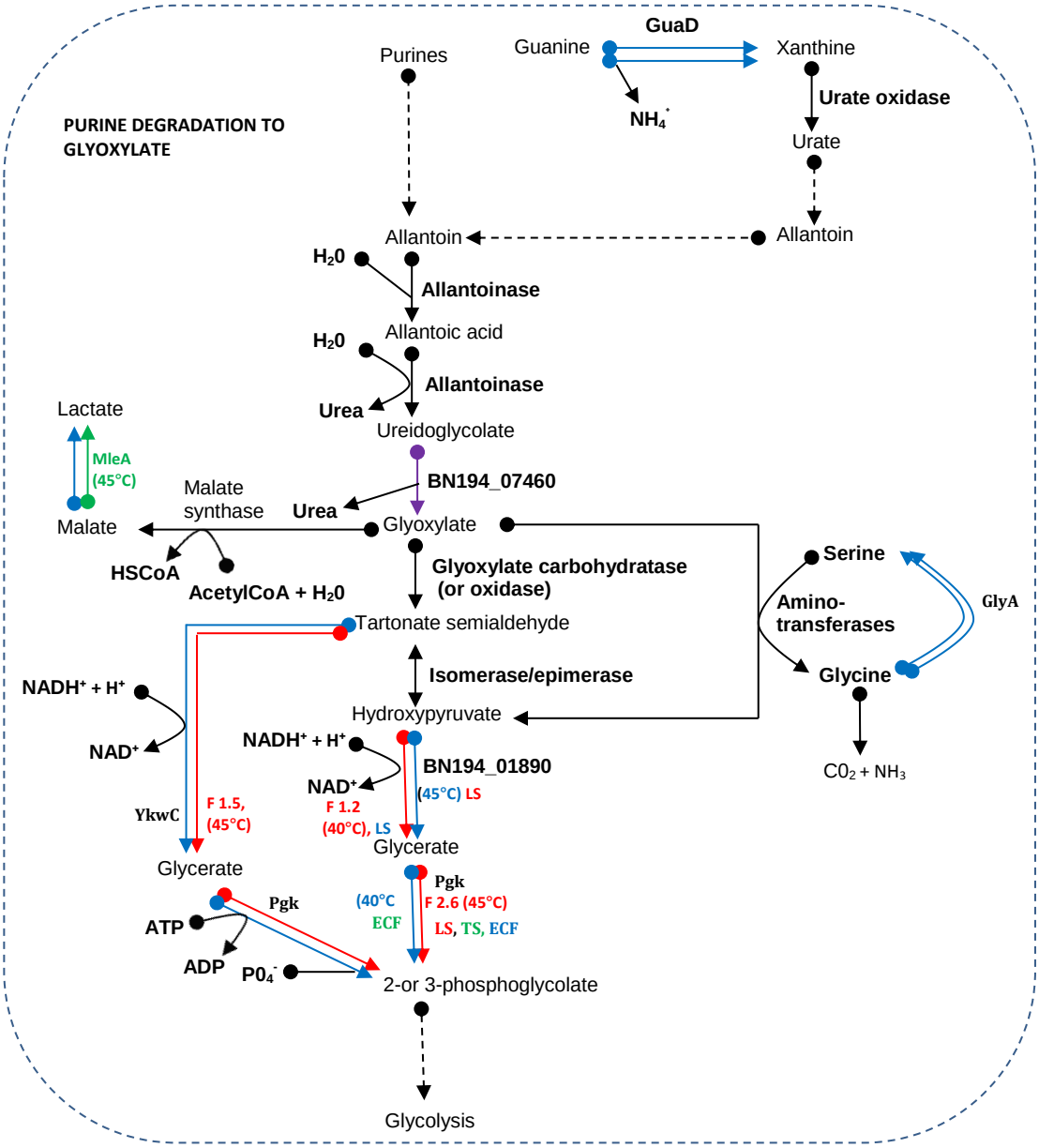


Figure 7. 9. Metabolic map illustrating proposed purine degradation for glyoxylate synthesis in *L. casei* GCRL163 under prolonged heat stress. GuaD, nucleoside deaminase; BN194_07460, ureidoglycolate lyase; BN194_01890, D-glycerate dehydrogenase/hydroxypyruvate reductase family protein; Pgk, phosphoglycerate kinase; YkwC, 3-hydroxyisobutyrate dehydrogenase family protein; GlyA, serine hydroxymethyltransferase; and MleA, malate dehydrogenase.

(NB: serine becomes hydroxypyruvate and glyoxylate becomes glycine).

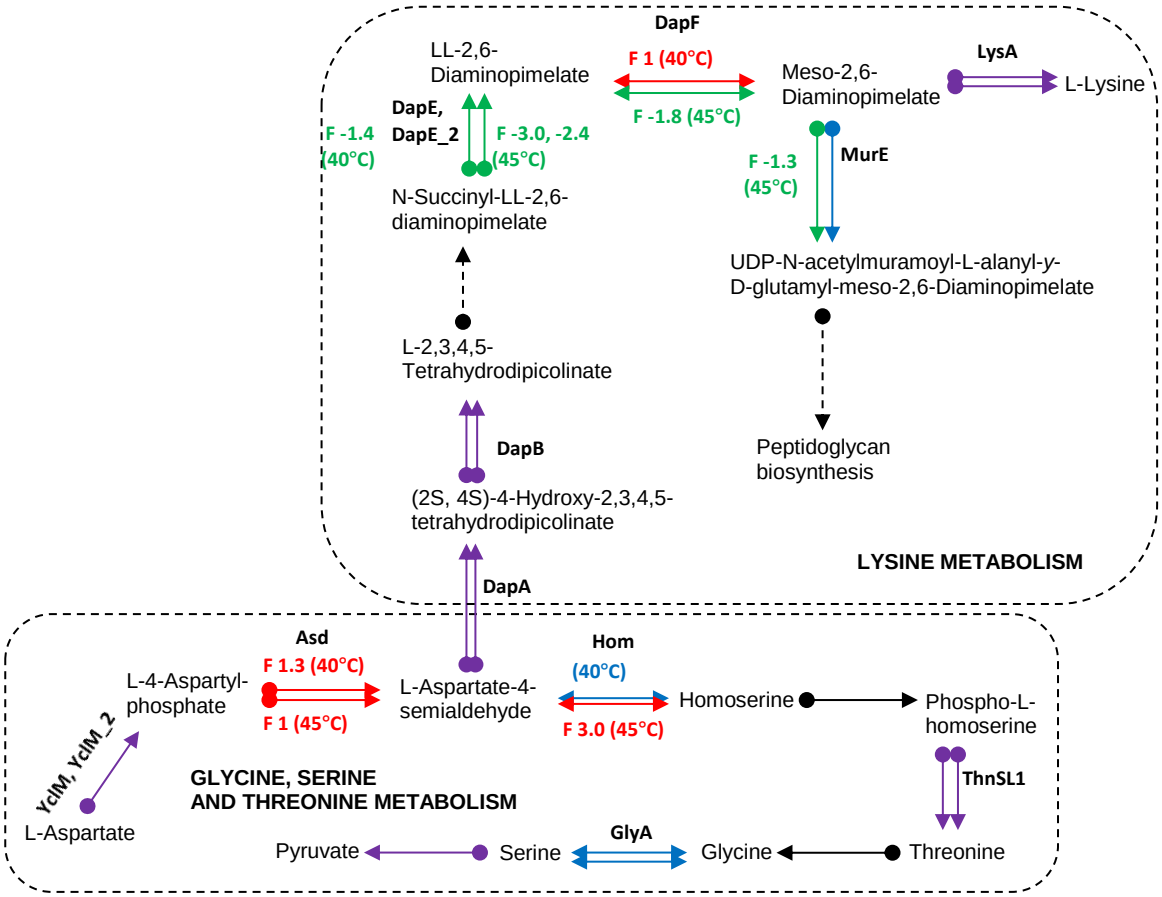


Figure 7. 10. Metabolic map of the proteins involved in the amino acid metabolism (glycine, serine and threonine) in *L. casei* GCRL163 under prolonged heat stress. DapE, DapE_2, succinyl-diaminopimelate desuccinylase; DapF, diaminopimelate epimerase; MurE, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase; GlyA, serine hydroxymethyltransferase; Asd, aspartate-semialdehyde dehydrogenase; and Hom, homoserine dehydrogenase.

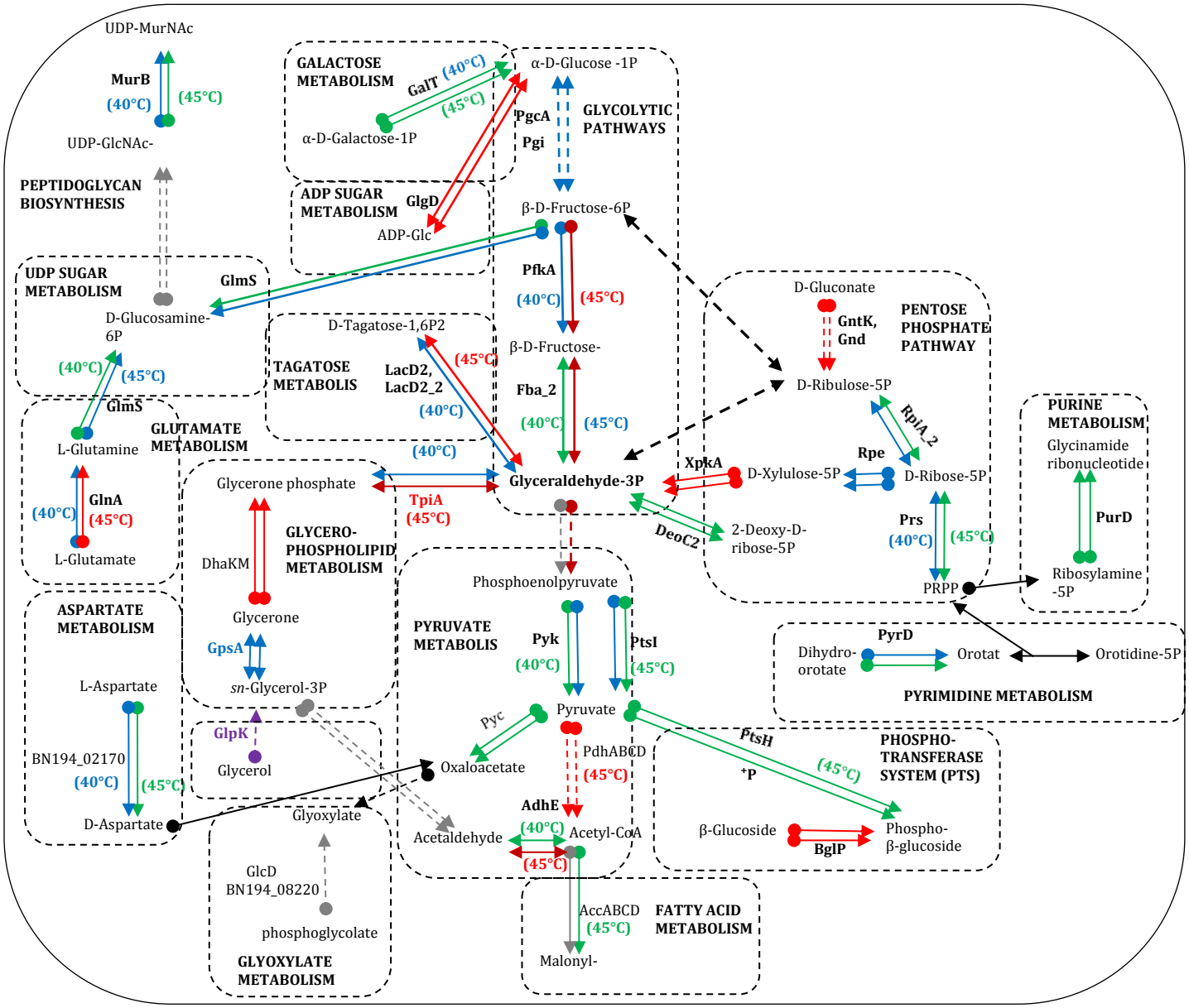


Figure 7. 11. Schematic overview of the metabolic map of *L. casei* GCRL163, illustrating relationship among key metabolic pathways under prolonged heat stress. MurB, UDP-N-acetylenolpyruvoylglucosamine reductase; GalT, galactose-1-phosphate uridylyltransferase; GlgD, glucose-1-phosphate adenylyltransferase; GlmS, glucosamine--fructose-6-phosphate aminotransferase; LacD2, LacD2_2, tagatose 1,6-diphosphate aldolase 2; PgcA, phosphoglucomutase; Pgi, glucose-6-phosphate isomerase; PfkA, 6-phosphofructokinase; Fba_2, fructose-bisphosphate aldolase; GlnA, glutamine synthetase; BN194_02170, aspartate racemase; DhaKM, dihydroxyacetone kinase complex; GpsA, glycerol-3-phosphate

dehydrogenase; BN194_8220, HAD-like hydrolase superfamily protein phosphoglycolate phosphatase-like; Pyc, pyruvate carboxylase; TpiA, triosephosphate isomerase; Pyk, pyruvate kinase; PdhABCD, Pyruvate dehydrogenase complex; AccABCD, acetyl-CoA carboxylase carboxyl transferase complex; PtsH, phosphocarrier protein HPr; PtsI, phosphoenolpyruvate-protein phosphotransferase; PyrD, dihydroorotate dehydrogenase A (fumarate); PurD, phosphoribosylamine-glycine ligase; Prs1, ribose-phosphate pyrophosphokinase 1; Rpe, ribulose-phosphate 3-epimerase; RpiA_2, ribose-5-phosphate isomerase A; XpkA, xylulose-5-phosphate phosphoketolase; GntK, gluconokinase; and Gnd, 6-phosphogluconate dehydrogenase.

CHAPTER 8

GENERAL CONCLUSION AND FUTURE DIRECTIONS

8.1 General conclusion

The work reported in this thesis investigated prolonged heat stress responses that could underpin physiological and molecular mechanisms that enable *Lactobacillus* spp. to adapt and function as probiotic and starter bacteria. Impact of prolonged heat stress on cell adhesion was investigated with potential novel adhesins identified. The key approach adopted in the study involved the use of proteomic tools to identify and analyse protein fractions from *L. casei* GCRL163. The protein fractions included, CFEs (containing largely cytoplasmic proteins), LS and TS extracts (cell surface proteins) and ECF precipitates (proteins secreted into the growth medium), obtained from *L. casei* GCRL163 cells, cultured under strictly controlled growth conditions using bioreactors that were mostly maintained at 30°C, 35°C, 40°C, 45°C and pH of 6.5. Modulations of the proteins in response to prolonged heat stress were investigated by comparing protein expressions in mid-exponential-phase cells at growth temperatures of 35°C, 40°C, and 45°C to 30°C. The selection of 30°C as the basal growth temperature and harvesting of cells at mid-exponential growth phase were some of the measures taken to reduce cell lysis that could impact on the isolation of cell surface proteins, as cell lysis was observed to increase with higher temperature (Chapter 4).

Furthermore, several prior studies in our laboratory, involving starvation and acid stress analyses with *L. casei* GCRL163, were conducted at 30°C to avoid heat stress, so data collected for cell extracts at 30°C in the present study would allow comparison with prior research and contribute to establishing broader data sets to determine the reproducibility of proteomic analyses between researchers and across time. Measures used to reduce artefacts

due to cell lysis included, the use of sucrose as an osmotic protectant and gentle handling of the cells during surface protein extraction. Protein abundances at growth temperature of 40°C, which is close to the optimal temperature of 37°C for the strain, reflect an induction of stress responses vital for cellular adaptation while cells at 45°C demonstrate some stress responses similar to stationary-growth-phase stress responses and a downregulation of several proteins (Chapter 3). This is consistent with the observed reduced biomass and early entrance into stationary growth phase that characterize cells cultured at 45°C, demonstrating a state of extreme heat stress and metabolic impairment.

Three complementary methods, TS, LS and ECF precipitation, were applied to obtain surface proteins and peptides from *L. casei* GCRL163 whole cells. Several proteins detected in these fractions differed not only in expressions but also in numbers, which demonstrated the importance of using multiple approaches to detect cell surface proteins. The analysis of the sub-proteome fractions from *L. casei* GCRL163 whole cells was quite challenging as protocols used to recover surface proteins in S-layer LAB, following lithium chloride extraction, were not effective in *L. casei* GCRL163. Johnson *et al*. (2016) had previously reported that S-layers, which formed an important structure that anchored secreted proteins to the cell surface in the *L. acidophilus* group, were not produced by *L. casei.* Previous extraction of surface proteins from *L. casei* treated with LiCl was reported to generate few proteins, most of which were cytoplasmic proteins (Johnson *et al*. 2016). To tackle this challenge, ethanol precipitation procedures were applied, which efficiently recovered the surface protein fraction following LiCl-sucrose treatment. The procedure was compatible with nanoLC-MS/MS and could be applied to bacteria with no S layer proteins.

The present study has demonstrated that culturing of cells at increasing temperatures resulted in an increased number of proteins impacted by prolonged heat stress, as 3 proteins, 285 proteins and 515 proteins of the total 773 filtered proteins in the CFEs were significantly impacted at 35°C, 40°C and 45°C respectively relative to growth at 30°C (Chapter 3). Several of these proteins are related to different cellular processes that are central to the adaptive mechanisms of prolonged heat stress. Stress sensors, including signal transducers, molecular chaperones, proteases, sensor kinases and transcriptional regulators were differentially expressed. Although, some of the sensor kinases, such as histidine kinase domain-containing proteins VanS and VanR, were not differentially modulated, LiaR was repressed in the CFE and LS fractions. OmpR/PhoB-type domain-containing proteins, including response regulators YycF_2, BceR_2 and universal stress protein UspA were up-regulated and could be important for transducing signals during thermal stress. Noteworthy, YycF are highly conserved and specific to low G+C Gram-positive bacteria such as *B. subtilis* and *S. aureus*, where they play central roles in cell wall metabolism and cell viability (Bisicchia *et al*. 2007; Dubrac *et al*. 2007). As a result of this, novel antimicrobial agents targeting YycF have been considered attractive (Gotoh *et al*. 2010). The expression of YycF family protein in *L. casei* GCRL163 in response to prolonged heat stress suggests that an inhibitor of YycF may impact the ability of the strain to cope with growth at elevated temperature.

Key regulators of PHSR in *L. casei* GCRL163 at proteome level were also reported in the study. These regulators, including transcriptional repressor HrcA, RNA polymerase σ^A and other core subunits of RNA polymerase α, β, β', δ, ω showed differential expression at different growth temperatures. However, transcriptional repressor CtsR and alternative σ factors (σ^{24}, σ^{32} and σ^{54}) and EpuA were not detected despite their presence in the genome of the strain (Nahar *et al*. 2017). The involvement of alternative sigma factors in heat stress

response has not been experimentally demonstrated in *L. casei*. However, alternative sigma (H)-like factor played minor roles in starvation stress response in *L. sakei* (Schmid *et al*. 2012). Interestingly, ribosome hibernation promoting factor Hpf with σ^{54} modulation domain was detected. The cytoplasmic protein Hpf is associated with ribosome hibernation during stress conditions in *E. coli* (Ueta *et al*. 2008) and ribosome dimerization in *B. subtilis* (Akanuma *et al*. 2016). These findings were unexpected considering the important roles played by alternative σ factors in initiating transcription and activating various adaptive responses in bacteria (Österberg *et al*. 2011). The absence of the transcriptional repressor CtsR in all the protein fractions and at all the growth temperatures might suggest limited roles of CtsR in the regulation of Clp system during prolonged heat stress in *L. casei* GCRL163. Similar to non-detection of CtsR in *L. casei* GCRL163 under prolonged heat stress, the repressor CtsR was not differentially detected in *L. casei* BL23 under bile stress (Alcantara and Zuniga 2012). CtsR is responsible for the negative regulation of the Clp system and other molecular chaperones in Gram-positive bacteria (Derré *et al*. 1999). Furthermore, σ^{B} was not detected in this study but proteins, such as Asp23 domain proteins (Asp23_2, YqhY and putative protein BN194_17970), YhjA, Hpf, and BN194_28350 (DgaF), showed varied differential expressions. Orthologs of these proteins are under the regulatory roles of σ^{B} in *B. subtilis* (Müller *et al*. 2014).

Several differentially abundant proteins related to other key functions and associated with PHSR in the strain were detected. In the CFEs, proteins associated with membrane bioenergetics and protein folding and turnover were consistently up-regulated at growth temperatures of 35°C, 40°C and 45°C relative to 30°C, proteins involved in phosphotransferase systems and ABC-type transporter systems were up-regulated only at 40°C and 45°C, while carbohydrate-related metabolism and central glycolytic and

intermediary pathways were enhanced specifically at 45°C. Most highly up-regulated proteins in the CFEs across all the growth temperatures relative to 30°C included 18 kDa α-crystalline Hsp20 domain acid shock protein BN194_29440 and non-chaperone protein hydroxyethylthiazole kinase ThiM. These proteins ThiM and 18 kDa α-crystalline BN194_29440 showed increasingly induced expressions as growth temperature increased and they could be used as biomarkers of heat stress, like the chaperones GroES-GroEL and DnaK-GrpE complexes, in the strain. Other highly over-expressed proteins in the CFEs included DnaJ and MetE at 35°C, ABC transporters DppE_3 and BN194_19800 at 40°C and, FruA_3 and glycosyltransferase family protein BN194_30000 at 45°C growth temperatures.

In the LS fractions of the cell surface proteins, the most over-expressed proteins at 35°C and 40°C included proteins involved in lipid-related metabolism (FabK, BN194_01080, AcpP_2, and BN194_22500) and ABC transporter BN194_25870. At 45°C growth temperature, hypothetical proteins BN194_15370 and BN194_11770, PTS EIIB domain-containing protein BN194_28560, and glycosyltransferase BN194_13290, were the most up-regulated. Some proteins were suppressed beyond detection in the 30°C control but were abundantly expressed at higher temperatures in the LS fractions. They included uncharacterized protein isoforms BN194_27750/BN194_27760, a putative α-L-fucosidase, PTS proteins BN194_07420 and LevE_4, putative ABC hemin import ATP-binding protein HrtA_2 and uncharacterised protein BN194_20240. In the TS protein fraction, the most up-regulated proteins at 35°C and 40°C included the CHAP/SibA domain-containing cell wall hydrolase BN194_00240, septum site-determining protein DivIVA, heat-shock protein serine protease HtrA/DegP, ribosomal protein RplX and NlpC/P60 domain-containing protein BN194_21500. At 45°C, XpaK involved in D-xylulose catabolism, PspC domain-containing protein YthC, ribosomal proteins RplW and RpsQ, and ATP-dependent Clp protease ClpP_2

became significantly more abundant. The majority of these up-regulated proteins in the CFEs and cell surface fractions are related to substrate uptake through the PTS- and ABC transporter systems, ribosomal proteins, cell wall hydrolysis, protein folding and turnover, exopolysaccharide catabolism and adhesins.

Several other proteins, which were found to be most abundantly expressed at the cell surface and secreted into the growth medium, could play key roles in PHSR and contribute to the strain functionality. The majority of these proteins are glycolytic enzymes, ribosomal proteins, chaperones, cell wall hydrolases, peptidases and autolysins. For instance, in the LS fractions, the most abundantly expressed proteins at all growth temperatures were the glycolytic proteins (Gap, Eno, Pyk and Pgk), elongation factor Tuf, DNA-binding protein Hup and chaperone GroEL. In the TS fractions, the most abundant proteins included glycolytic proteins (Pgk, Pyk, and Eno), ribosomal proteins (RplL, RpsA, RplB and RplD), molecular chaperones (DnaK and GroEL), elongation factors Tuf and Fus, DNA-binding protein Hup and general secretory pathway protein SecA. In the ECF fractions, most abundant proteins included several functionally uncharacterised peptidases and hydrolases putatively involved in peptidoglycan turnover (BN194_00240, BN194_02560, BN194_02820, BN194_21500, and BN194_23630), putative autolysins (BN194_02430 and YrvJ), and cytoplasmic proteins (GroEL, Tuf). The majority of most abundantly and differentially expressed proteins in the CFEs and cell surface fractions are related to stress responses (molecular chaperones and proteases), energy production (glycolytic enzymes), generation of additional carbon and nitrogen sources and cell division (cell wall hydrolases, peptidases, glycosyltransferases and autolysins), substrate uptake (PTS- and ABC transporter systems), protein synthesis (ribosomal proteins), cell membrane maintenance (fatty acid and lipid metabolism) and cell adhesion (adhesins and moonlighting proteins).

In heat stress, the ATP usage increases due to greater protein chaperone activity (Rothman & Schekman 2011). Establishing a network of metabolic and cellular mechanisms to generate more cellular energy becomes important to the bacteria (Di Cagno *et al*. 2006). This is consistent with the abundant and up-regulated expressions of proteins in glycolysis and intermediary-related metabolism, which are vital for generating more cellular energy in the form of ATP during prolonged heat stress. However, in *Lactobacillus* spp., the ATP generated from the fermentation metabolism through glycolysis and other metabolic pathways, such as pyruvate metabolism, is not sufficient to meet up with the growth demands, hence alternative energy production is often required (Pessione *et al*. 2010). The alternative energy production can involve amino acid decarboxylation, malate decarboxylation and/or arginine deamination (Pessione *et al*. 2010). In this study, malate dehydrogenase MleA, involved in the reduction of malate to L-lactate, was repressed. Other decarboxylases, including enzymes involved in the decarboxylation of histidine to histamine, tyrosine to tyramine, ornithine to putrescine, lysine to cadaverine and glutamate to GABA (Santos 1996), were not detected. These findings suggest that *L. casei* GCRL163 depends mainly on sugar fermentation at mid-exponential growth phase for metabolic energy rather than alternative energy source during heat stress. To cope with prolonged heat stress, additional carbon sources must be available to ensure a continuous supply of energy.

The overexpression of several proteins in the PTS systems involved in the uptake of a variety of sugars other than glucose suggested other carbon sources were mobilized during prolonged heat stress. For instance, proteins involved in β-glucoside uptake (BglP), mannose uptake (ManY, BN194_29710; ManX, BN194_29720 and ManZ, BN194_29700) and galactose uptake (BN194_07430) were over-expressed at 40°C and 45°C while GmuB

involved in cellobiose uptake was moderately up-regulated at 45°C. It is worth mentioning that the NCBI and RAST annotations of the proteins involved in the PTS and ABC transporter systems might not necessarily signify specific functionality, as a protein can transport more than one sugar. The additional carbon and nitrogen sources were possibly generated from peptidoglycan, exopolysaccharide, lipid and nucleotide degradation during growth. Cell wall hydrolases, peptidases, glycosyltransferases and autolysins, were abundantly expressed particularly at 40°C with repressed expression at 45°C. LytR family proteins are responsible for the maintenance of cell wall structures through the regulation of autolysin (Chatfield *et al*. 2005). An enhanced expression of LytR family proteins (cell wall-anchored proteins LytR_3 and LytR_4, possessing LytR_CpsA_Psr and LytR_CpsA domain respectively) at 40°C and repressed expression at 45°C was therefore consistent with the expression pattern of proteins associated with cell wall hydrolysis. The generation and uptake of endogenously derived sugars during prolonged heat stress are understandable as these sugars are part of cell envelope constituents and can be phosphorylated and used as substrates by *Lactobacillus* spp (Pessione *et al*. 2010). The phosphorylated substrates can directly enter into the glycolytic pathways and be used to generate energy, thereby saving the bacterial cells ATP in the process (Cohen *et al*. 2006).

However, a preference for glucose utilization was observed in this study, as proteins associated with the catabolism of carbohydrates, including GalE_2, GalT, BgaL17, Cap4C, AgL and BN194_07390 involved in the galactose, fructose and sucrose metabolism, were either down-regulated or not differentially expressed, despite the upregulation of proteins associated with a variety of carbohydrate uptake. These findings coincide with the up-regulated synthesis of several enzymes in the glycolytic pathway, especially at 45°C. The glycolytic precursors induce HPr phosphorylation at the serine residue at position 46 with the

resultant P-Ser-HPr interacting with catabolite control protein A (CcpA) to prevent catabolism of other carbohydrates (Papadimitriou *et al*. 2016). Moreover, a network of metabolic pathways was activated to channel biosynthetic precursors into the glycolytic pathways to generate additional carbon sources and enhance energy production in order to meet high-energy demand activities during prolonged heat stress. Notably, growth temperature of 45°C induced an upregulation of proteins involved in the nucleotide sugar metabolism (NagA, NagB and ManD), tagatose metabolism (LacC, LacD2, LacD2_2 and PfkA) and Entner-doudoroff pathway (GntK and Gnd while the expression of proteins RpiA_2, RbsK and Prs1 for generating PRPP which is a substrate for purine, pyrimidine and histidine metabolism were repressed). Moreover, phosphoketolase XpkA, involved in the generation of glycealdehyde-3P from xylulose-5P and TpiA which could channelled gycerone phosphate from the glycerophospholipid metabolism into the synthesis of glyceraldehyde-3P in the glycolytic pathway, were over-expressed. Upregulation of DhaM and DhaK converting glycerone to the glycerone phosphate could suggest generation of carbon source from lipid degradation. There was also an indication, via unknown mechanisms, that nucleotide degradation occurred during prolonged heat stress, channelling carbon into glyoxylate pathways.

The expression of proteins involved in the synthesis of peptidoglycan precursors depicted that *L. casei* GCRL163 was not enhancing peptidoglycan synthesis but rather maintaining the integrity of the cell envelope at the high temperature of 40°C close to the optimum while 45°C prolonged heat stress repressed proteins such as Pmi, GlmU, GlmS, MurA2 and MurB, suggesting reduced peptidoglycan synthesis. Moreover, the overexpression of autolysins at 35°C and 40°C including, cell wall hydrolase BN194_02430 with a peptidoglycan autolysin glycoside-hydrolase-lysozyme domain and cell wall

hydrolase/autolysin with cat/SH3-like domain BN194_17200, further suggested that cell wall maintenance, cell division and peptidoglycan autolysis were important as PHSR in the strain. Furthermore, several proteins involved in the fatty acid metabolism were moderately over-expressed at 40°C but markedly repressed at 45°C. Analysis of the fatty acid composition of the strain at different growth phases revealed that oleic acid was the principal unsaturated fatty acid with vaccenic acid greatly reduced. The oleic acid decreased from exponential to stationary phase and also decreased as growth temperatures were elevated. Novel cyclopentenyl moieties produced in all the growth phases were possibly synthesized from the cyclization of oleic acid with decreased synthesis at 45°C. The reduced production of the oleic acid and the enzymes involved in the FA biosynthesis at 45°C might be a conserved effort by the heat adapted cells to preserve energy. The insertion of the double bond, into oleic and vaccenic acids, possibly occurs through FabZ at the time of chain elongation. The accumulation of vaccenic acid, which did not occur at 45°C, suggested that the cells suppressed fatty acid synthesis early in the growth phase which impaired vaccenic acid production. However, existing oleic acid is modified to cyclo-derivatives.The fatty acid biosynthesis is an energy intensive process and the cells have the capacity to adjust existing fatty acids thereby conserving energy in synthesising new fatty acids (Jerga & Rock 2009). The regulation of fatty acid biosynthesis in *S. pneumoniae* is associated with YycF which binds to FabT region (a fatty acid biosynthesis repressor) and inhibits FabT transcription (Mohedano *et al*. 2016). These findings are consistent with the non-differential expression of YycF_2 at 40°C and an upregulation at 45°C. Other energy conserving cellular processes could be related to downregulation of several proteins in the nucleotide and amino acid metabolisms. For instance, in the CFEs, the most repressed proteins at 35°C were PepC, PepT, Cap4C, Sph and GlnA_2 while PurC, PurD, and PurH, involved in purine biosynthesis and PepC were the most down-regulated proteins at 40°C and 45°C. Interestingly, the *pur*

operon repressor PurR_2, associated with the regulation of purine biosynthesis was repressed at 40°C and 45°C in the CFEs and also inhibited in the TS fraction at 45°C.

Furthermore, upregulation of cell wall hydrolases (50kDa NlpC/P60 SLAP domain-containing protein BN194_02820 and 43 kDa CHAP/SibA domain-containing protein BN194_00240) and abundance of other proteins (PspC-domain protein YthC and moonlighting proteins such as glycolytic enzymes enolase, Gap, LacC and LacD_2) at the cell surface suggested that prolonged heat stress might improve cell adhesion and probiotic functionality in LAB. The 50kDa BN194_02820 NlpC/P60 and 43 kDa BN194_00240 are homologous to p75 and p40 secreted proteins of *L. rhamnosus* GG respectively. The p75 and p40 have been associated with anti-apoptotic and cell protective activities on intestinal epithelial cells and reduced adverse impact of hydrogen peroxide on epithelial barrier function (Seth *et al*. 2008; Yan *et al*. 2007; Yan & Polk 2002; Yan & Polk 2012). In cells at stationary growth phase, several proteins associated with cell adhesion, including the cell wall hydrolases 50kDa BN194_02820 NlpC/P60 and 43 kDa BN194_00240 were also detected at the cell surface. The majority of the glycolytic enzymes detected with abundant expressions at the cell surface during prolonged heat stress are associated with moonlighting functions, contributing to host-bacterial interactions (Wang *et al*. 2013).

The binding assays performed on cells harvested at mid-exponential growth phase to HT-29 cells revealed the impact of prolonged heat stress on the binding capacity of *L. casei* GCRL163. The use of cells at mid-exponential-growth phase supplemented with additional 1% glucose in the bioreactors instead of the stationary growth phase was to preclude the influence of other stressors especially nutrient starvation which characterized stationary growth phase. It is worth mentioning though that 2M NaoH added to maintain the pH may

impact on the stress responses of the strain during prolonged heat stress. Adhesion to both chloroform and diethyl ether was higher at growth temperatures that were lower (30°C) and higher (40°C and 45°C) than 35°C. The findings demonstrated the non-acidic nature of *L. casei* GCRL163 cell surface with the cells becoming more hydrophilic at growth temperature close to the optimum compared to lower or higher growth temperatures, suggesting that the cell binding capacity to host cells could be impacted by growth at different temperatures. Further analysis demonstrated that *L. casei* GCRL163 showed moderate adhesion to HT-29 cells compared to other LAB strains, with prolonged heat stress at 40°C resulting in an increased adhesion compared to 30°C in cells maintained at pH of 6.5 and 4.5. Moreover, mid-exponential-growth phase *L. casei* GCRL163 cultured at pH 6.5 adhered more to the HT-29 cells than cells cultured at pH 4.5. These findings clearly demonstrated that prolonged heat stress of *L. casei* GCRL163 led to an improved adhesion to HT-29 cells.

This study revealed several other uncharacterized proteins with unknown functions, many of which were differentially and abundantly expressed. Notably, Flik family flagellar hook-length control proteins BN194_29420 and BN194_06730 and 15 kDa protein with gene locus BN194_10000 with the domain of export chaperone SecB demonstrated differential abundance during prolonged heat stress. Some of these proteins possessed gene ontology (GO) annotations while others could only be assigned putative identification using BLASTN (Consortium 2014).

8.2 Future directions

8.2.1 Detection of more proteins encoded in the genome

In this study, approximately 36% of proteins encoded on the genome of *L. casei* GCRL163 were detected in the CFEs, out of which about 30% of the encoded proteins were considered valid following stringent filtering creteria (see Chapter 2.3.1.8). A total of 2,938 genes and 2,807 coding genes are present in the genome of *L. casei* GCRL163 (Nahar *et al*. 2017). Optmizing extraction conditions using different extraction methods may increase the number of protein detected and improve current knowledge on stress responses.

8.2.2 Non-detection of key regulatory proteins

Non-detection of transcriptional repressor CtsR and alternative sigma factors in the CFE, LS, TS and ECF protein fractions at any growth temperatures or stage of growth could be due to several factors, ranging from proteomic analysis limitations, undetectable LFQ intensities, non-induction or suppression by heat stress and degradation by proteases. CtsR repressor can be degraded by ClpCP and ClpEP protease complexes such that the suppression of proteins under its control is inhibited (Krüger *et al*. 2001). Integrating transcriptomics with proteomics can provide further insight into the unknown mechanisms. Transcriptomics is a technique used for investigating transcriptome, including all the coding and non-coding RNA molecules and may involve either microarray or RNA sequencing. Microbial RNA-seq experiments allow annotation and quantification of comprehensive transcripts in bacteria (Creecy & Conway 2015). The RNA-seq analysis allows confident identification of both high and low expressors in a single bacteria and unbiased strand-specific identification of novel transcripts compared to microarrays. The procedure may involve extraction of cellular RNA which is converted to cDNA for preparation of sequencing libraries. The sequence reads are then mapped back to the reference genome to provide qualitative and quantitative transcript data. The sequencing can be conducted under different growth and stress conditions, to determine if these regulatory proteins are expressible at transcriptional level.

8.2.3 Moonlighting protein transport via microvesicles

Several cytoplasmic proteins were relatively abundant at the cell surface and could be due to moonlighting phenomenon, cell lysis or microvesicles carrying cytoplasmic proteins to the cell surface. Microvesicles have recently be linked to the the transport of cytoplasmic proteins to the cell surface in *L. casei* (Domínguez-Rubio *et al*. 2017). Previous work in our laboratory involving surface protein extraction using lithium chloride observed only few proteins at the cell surface of *L. casei* GCRL163 after using dialysis tubing to concentrate the protein fractions. It is possible that any generated microvesicle has been extracted by lithium chloride and lost during sample preparations and handlings such as concentrating proteins using dialysis tubing. Interestingly, when the concentrating by dialysis tubing procedure was replaced with concentrating by precipitation using ethanol in the current study, several proteins were detected including the orthologs of the microvesicle content. This suggests that microvesicle production is a possibility in *L. casei* GCRL163. The microvesicles might have been dissolved by the precipitating agent (ethanol), thereby allowing the contents to be accessible. Further research will be needed to investigate the production of microvesicles in *L. casei* GCRL163. Investigating the contents of the microvesicles, the impact of stress conditions on the composition of the contents and how this can be applied to improve probiotic functionality will provide interesting opportunities for boosting host health.

8.2.4 Investigating functions of hypothetical proteins

Several proteins were detected in different protein fractions as uncharacterized proteins with hypothetical functions. Some of these proteins demonstrated differential expressions that suggested they could be vital in PHSR. One of these proteins is an uncharacterized 15 kDa protein BN194_10000 which possesses the domain of export

chaperone SecB. This SecB-like protein was abundantly expressed at 30°C and showed repressed expression at 40°C and 45°C in *L. casei* GCRL163. We suggested that the SecB-like protein did not actively participate in post-translational secretion under prolonged heat stress in the strain, a role possibly played by the up-regulated chaperones. SecB domain is conserved in several genomes of *L. casei* strains (Consortium 2016) and SecB is involved in post-translational secretion in Gram-positive and Gram-negative bacteria (Randall 1992). Procedures involving gene deletion may shed more light on the involvement of this protein in post-translational secretion under different stress conditions.

8.2.5 Investigating prolonged heat stress responses in other strains of *L. casei*

Previous work in our laboratory, involving different *L. casei* strains, have demonstrated strain variation not only in growth pattern but also in protein expression under acid stress and at different growth phases. Investigating prolonged heat stress in other *L. casei* strains and other closely related bacteria will widen the knowledge of how strain variation impacts on prolonged heat stress response in *Lactobacillus*.

8.2.6 Investigating adaptation responses in *L. casei* GCRL163 under different stress conditions

Some proteins, such as 18 kDa α-crystalline Hsp20 domain acid shock protein BN194_29440 and non-chaperone protein hydroxyethylthiazole kinase ThiM, were increasingly up-regulated as growth temperatures increased, in similar pattern as several of the chaperones. Others proteins, including some regulatory proteins of PHSR, were also up-regulated. Some of these proteins were suggested could be used as biomarkers of prolonged heat stress and the expressions of several other proteins were considered to be specific to

prolonged heat stress in *L. casei* GCRL163. Investigating adaptation responses under different stress conditions will shed more light into the contention.

In general, the results reported in this thesis have shed more light on prolonged heat stress response in *L. casei* GCRL163. The results further increase the current knowledge on regulation of proteins involved in the regulation of PHSR and metabolic pathway modifications under prolonged heat stress. The findings contributed to better understanding of the impact of prolonged heat stress on host-bacteria interactions.

REFERENCES

Adlerberth, I, Ahrne, S, Johansson, M-L, Molin, G, Hanson, LA & Wold, AE 1996, 'A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29', *Applied and Environmental Microbiology*, vol. 62, no. 7, pp. 2244-2251.

Akanuma, G, Kazo, Y, Tagami, K, Hiraoka, H, Yano, K, Suzuki, S, Hanai, R, Nanamiya, H, Kato-Yamada, Y & Kawamura, F 2016, 'Ribosome dimerization is essential for the efficient regrowth of *Bacillus subtilis*', *Microbiology*, vol. 162, no. 3, pp. 448-458.

Al-Awqati, Q 1986, 'Proton-translocating ATPases', *Annual Review of Cell Biology*, vol. 2, no. 1, pp. 179-199.

Alcántara, C, Revilla-Guarinos, A & Zúñiga, M 2011, 'Influence of two-component signal transduction systems of *Lactobacillus casei* BL23 on tolerance to stress conditions', *Applied and Environmental Microbiology*, vol. 77, no. 4, pp. 1516-1519.

Alcantara, C, & Zuniga, M, 2012, 'Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23', *Microbiology*, vol. 158, no. 5, pp. 1206-1218.

Al-Naseri, A, Bowman, JP, Wilson, R, Nilsson, RE & Britz, ML 2013, 'Impact of lactose starvation on the physiology of *Lactobacillus casei* GCRL163 in the presence or absence of tween 80', *Journal of Proteome Research*, vol. 12, no. 11, pp. 5313-5322.

Alekshun, MN & Levy, SB 1999, 'The mar regulon: multiple resistance to antibiotics and other toxic chemicals', *Trends in Microbiology*, vol. 7, no. 10, pp. 410-413.

Amblee, V & Jeffery, CJ 2015, 'Physical Features of Intracellular Proteins that Moonlight on the Cell Surface', *PLoS ONE*, vol. 10, no. 6, p. e0130575.

Anderson, NL & Anderson, NG 1998, 'Proteome and proteomics: new technologies, new concepts, and new words', *Electrophoresis*, vol. 19, no. 11, pp. 1853-1861.

Angelis, M, Calasso, M, Cavallo, N, Di Cagno, R & Gobbetti, M 2016, 'Functional proteomics within the genus *Lactobacillus*', *Proteomics*, vol. 16, no. 6, pp. 946-962.

Antikainen, J, Kupannen, V, Lähteenmäki, K & Korhonen, TK 2007, 'pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids', *Journal of Bacteriology*, vol. 189, no. 12, pp. 4539-4543.

Anuchin, A, Goncharenko, A, Demidenok, O & Kaprelyants, A 2011, 'Histone-like proteins of bacteria', *Applied Biochemistry and Microbiology*, vol. 47, no. 6, p. 580.

Arora, M & Baldi, A 2015, 'Regulatory categories of probiotics across the globe: A review representing existing and recommended categorization', *Indian Journal of Medical Microbiology*, vol. 33, no. 5, p. 2.

Aryantini, NPD, Kondoh, D, Nishiyama, K, Yamamoto, Y, Mukai, T, Sujaya, IN, Urashima, T & Fukuda, K 2017, 'Anchorless cell surface proteins function as laminin-binding adhesins in *Lactobacillus rhamnosus* FSMM22', *Fems Microbiology Letters*, vol. 364, no. 6, p. fnx056.

Aseev, L & Boni, I 2011, 'Extraribosomal functions of bacterial ribosomal proteins', *Molecular Biology*, vol. 45, no. 5, pp. 739-750.

Bagos, PG, Tsirigos, KD, Liakopoulos, TD & Hamodrakas, SJ 2008, 'Prediction of lipoprotein signal peptides in Gram-positive bacteria with a Hidden Markov Model', *Journal of Proteome Research*, vol. 7, no. 12, pp. 5082-5093.

Baker-Austin, C & Dopson, M 2007, 'Life in acid: pH homeostasis in acidophiles', *Trends in Microbiology*, vol. 15, no. 4, pp. 165-171.

Bandi, ZL & Mangold, HK 1969, 'Chromatography of lipids containing cyclopentenyl fatty acids', *Separation Science and Technology*, vol. 4, no. 1, pp. 83-88.

Bastin, G 2013, *On-line estimation and adaptive control of bioreactors*, vol. 1, Elsevier.

Båth, K, Roos, S, Wall, T & Jonsson, H 2005, 'The cell surface of *Lactobacillus reuteri* ATCC 55730 highlighted by identification of 126 extracellular proteins from the genome sequence', *Fems Microbiology Letters*, vol. 253, no. 1, pp. 75-82.

Bauerl, C, Perez-Martinez, G, Yan, F, Polk, DB & Monedero, V 2010, 'Functional Analysis of the p40 and p75 Proteins from *Lactobacillus casei* BL23', *Journal of Molecular Microbiology and Biotechnology*, vol. 19, no. 4, pp. 231-241.

Bäuerl, C, Pérez-Martínez, G, Yan, F, Polk, DB & Monedero, V 2010, 'Functional analysis of the p40 and p75 proteins from *Lactobacillus casei* BL23', *Journal of Molecular Microbiology and Biotechnology*, vol. 19, no. 4, pp. 231-241.

Beaufils, S, Sauvageot, N, Mazé, A, Laplace, J-M, Auffray, Y, Deutscher, J & Hartke, A 2007, 'The cold shock response of *Lactobacillus casei*: relation between HPr phosphorylation and resistance to freeze/thaw cycles', *Journal of Molecular Microbiology and Biotechnology*, vol. 13, no. 1-3, pp. 65-75.

Bell, K 1962, 'One-dimensional starch-gel electrophoresis of bovine skim-milk', *Nature*, vol. 195, no. 4842, p. 705.

Bellon-Fontaine, M-N, Rault, J & Van Oss, C 1996, 'Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells', *Colloids and Surfaces B: Biointerfaces*, vol. 7, no. 1, pp. 47-53.

Bender, GR & Marquis, RE 1987, 'Membrane ATPases and acid tolerance of *Actinomyces viscosus* and *Lactobacillus casei*', *Applied and Environmental Microbiology*, vol. 53, no. 9, pp. 2124-2128.

Berg, RD 1996, 'The indigenous gastrointestinal microflora', *Trends in Microbiology*, vol. 4, no. 11, pp. 430-435.

Bergonzelli, GE, Granato, D, Pridmore, RD, Marvin-Guy, LF, Donnicola, D & Corthésy-Theulaz, IE 2006, 'GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*', *Infection and Immunity*, vol. 74, no. 1, pp. 425-434.

Bernardeau, M, Vernoux, JP, Henri-Dubernet, S & Gueguen, M 2008, 'Safety assessment of dairy microorganisms: the *Lactobacillus* genus', *International Journal of Food Microbiology*, vol. 126, no. 3, pp. 278-285.

Bernhardt, J, Weibezahn, J, Scharf, C & Hecker, M 2003, *'Bacillus subtilis* during feast and famine: visualization of the overall regulation of protein synthesis during glucose starvation by proteome analysis', *Genome Research*, vol. 13, no. 2, pp. 224-237.

Beyer, NH, Roepstorff, P, Hammer, K & Kilstrup, M 2003, 'Proteome analysis of the purine stimulon from *Lactococcus lactis*', *Proteomics*, vol. 3, no. 5, pp. 786-797.

Beynon, RJ, Hammond, D, Harman, V & Woolerton, Y 2014, 'The role of proteomics in studies of protein moonlighting', *Biochemical Society Transactions*, vol. 42, no. part 6, pp. 1698-1703.

Bidart, GN, Rodríguez-Díaz, J, Monedero, V & Yebra, MJ 2014, 'A unique gene cluster for the utilization of the mucosal and human milk-associated glycans galacto-N-biose and lacto-N-biose in *Lactobacillus casei*', *Molecular Microbiology*, vol. 93, no. 3, pp. 521-538.

Bisicchia, P, Noone, D, Lioliou, E, Howell, A, Quigley, S, Jensen, T, Jarmer, H & Devine, KM 2007, 'The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*', *Molecular Microbiology*, vol. 65, no. 1, pp. 180-200.

Blackstock, WP & Weir, MP 1999, 'Proteomics: quantitative and physical mapping of cellular proteins', *Trends in Biotechnology*, vol. 17, no. 3, pp. 121-127.

Bolotin, A, Wincker, P, Mauger, S, Jaillon, O, Malarme, K, Weissenbach, J, Ehrlich, SD & Sorokin, A 2001, 'The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403', *Genome Research*, vol. 11, no. 5, pp. 731-753.

Boorsma, A, Foat, BC, Vis, D, Klis, F & Bussemaker, HJ 2005, 'T-profiler: scoring the activity of predefined groups of genes using gene expression data', *Nucleic Acids Research*, vol. 33, no. suppl_2, pp. W592-W595.

Boot, HJ, Kolen, C & Pouwels, PH 1995, 'Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species', *Journal of Bacteriology*, vol. 177, no. 24, pp. 7222-7230.

Borchers, AT, Selmi, C, Meyers, FJ, Keen, CL & Gershwin, ME 2009, 'Probiotics and immunity', *Journal of Gastroenterology*, vol. 44, no. 1, pp. 26-46.

Bordes, P, Cirinesi, A-M, Ummels, R, Sala, A, Sakr, S, Bitter, W & Genevaux, P 2011, 'SecB-like chaperone controls a toxin–antitoxin stress-responsive system in *Mycobacterium tuberculosis*', *Proceedings of the National Academy of Sciences*, vol. 108, no. 20, pp. 8438-8443.

Borukhov, S & Severinov, K 2002, 'Role of the RNA polymerase sigma subunit in transcription initiation', *Research in Microbiology*, vol. 153, no. 9, pp. 557-562.

Botes, M, Loos, B, van Reenen, CA & Dicks, LM 2008, 'Adhesion of the probiotic strains *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments', *Archives of Microbiology*, vol. 190, no. 5, pp. 573-584.

Bowman, JP, Hages, E, Nilsson, RE, Kocharunchitt, C & Ross, T 2012, 'Investigation of the *Listeria monocytogenes* Scott A acid tolerance response and associated physiological and phenotypic features via whole proteome analysis', *Journal of Proteome Research*, vol. 11, no. 4, pp. 2409-2426.

Braconi, D, Amato, L, Bernardini, G, Arena, S, Orlandini, M, Scaloni, A & Santucci, A 2011, 'Surfome analysis of a wild-type wine *Saccharomyces cerevisiae* strain', *Food Microbiology*, vol. 28, no. 6, pp. 1220-1230.

Bremer, E 2000, 'Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in *Bacillus subtilis*', *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, vol. 126, p. 17.

Brennan, M, Wanismail, B, Johnson, M & Ray, B 1986, 'Cellular damage in dried *Lactobacillus acidophilus*', *Journal of Food Protection*, vol. 49, no. 1, pp. 47-53.

Broadbent, JR, Larsen, RL, Deibel, V & Steele, JL 2010, 'Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress', *Journal of Bacteriology*, vol. 192, no. 9, pp. 2445-2458.

Broadbent, JR, Oberg, CJ, Wang, H & Wei, L 1997, 'Attributes of the heat shock response in three species of dairy *Lactobacillus*', *Systematic and Applied Microbiology*, vol. 20, no. 1, pp. 12-19.

Bron, PA, Marco, M, Hoffer, SM, Van Mullekom, E, De Vos, WM & Kleerebezem, M 2004, 'Genetic characterization of the bile salt response in *Lactobacillus Plantarum* and analysis of responsive promoters in vitro and in situ in the gastrointestinal tract', *Journal of Bacteriology*, vol. 186, no. 23, pp. 7829-7835.

Brooijmans, R, De Vos, W & Hugenholtz, J 2009, '*Lactobacillus plantarum* WCFS1 electron transport chains', *Applied and Environmental Microbiology*, vol. 75, no. 11, pp. 3580-3585.

Brown, L, Villegas, JM, Elean, M, Fadda, S, Mozzi, F, Saavedra, L & Hebert, EM 2017, 'YebC, a putative transcriptional factor involved in the regulation of the proteolytic system of *Lactobacillus*', *Scientific Reports*, vol. 7, no. 1, p. 8579.

Brown, NL, Stoyanov, JV, Kidd, SP & Hobman, JL 2003, 'The MerR family of transcriptional regulators', *FEMS Microbiology Reviews*, vol. 27, no. 2-3, pp. 145-163.

Buck, BL, Altermann, E, Svingerud, T & Klaenhammer, TR 2005, 'Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM', *Applied and Environmental Microbiology*, vol. 71, no. 12, pp. 8344-8351.

Bucka-Kolendo, J & Sokołowska, B 2017, 'Lactic acid bacteria stress response to preservation processes in the beverage and juice industry', *Acta Biochimica Polonica*, vol. 64, no. 3.

Bukau, B 1993, 'Regulation of the *Escherichia coli* heat-shock response', *Molecular Microbiology*, vol. 9, no. 4, pp. 671-680.

Bukau, B, Weissman, J & Horwich, A 2006, 'Molecular chaperones and protein quality control', *Cell*, vol. 125, no. 3, pp. 443-451.

Burne, RA & Marquis, RE 2000, 'Alkali production by oral bacteria and protection against dental caries', *Fems Microbiology Letters*, vol. 193, no. 1, pp. 1-6.

Butorac, A, Dodig, I, Bacun-Druzina, V, Tishbee, A, Mrvcic, J, Hock, K, Diminic, J & Cindric, M 2013, 'The effect of starvation stress on *Lactobacillus brevis* L62 protein profile

determined by de novo sequencing in positive and negative mass spectrometry ion mode', *Rapid Communications in Mass Spectrometry*, vol. 27, no. 9, pp. 1045-1054.

Capozzi, V, Arena, MP, Crisetti, E, Spano, G & Fiocco, D 2011, 'The *hsp16* gene of the probiotic *Lactobacillus acidophilus* is differently regulated by salt, high temperature and acidic stresses, as revealed by reverse transcription quantitative PCR (qRT-PCR) analysis', *International Journal of Molecular Sciences*, vol. 12, no. 8, pp. 5390-5405.

Capozzi, V, Fiocco, D, Weidmann, S, Guzzo, J & Spano, G 2012, 'Increasing membrane protection in *Lactobacillus plantarum* cells overproducing small heat shock proteins', *Annals of Microbiology*, vol. 62, no. 2, pp. 517-522.

Carvalho, AS, Silva, J, Ho, P, Teixeira, P, Malcata, FX & Gibbs, P 2004, 'Relevant factors for the preparation of freeze-dried lactic acid bacteria', *International Dairy Journal*, vol. 14, no. 10, pp. 835-847.

Castro-Borges, W, Dowle, A, Curwen, RS, Thomas-Oates, J & Wilson, RA 2011, 'Enzymatic shaving of the tegument surface of live schistosomes for proteomic analysis: a rational approach to select vaccine candidates', *PLoS Negl Trop Dis*, vol. 5, no. 3, p. e993.

Catherman, AD, Skinner, OS & Kelleher, NL 2014, 'Top down proteomics: facts and perspectives', *Biochemical and Biophysical Research Communications*, vol. 445, no. 4, pp. 683-693.

Cebrián, G, Condón, S & Mañas, P 2009, 'Heat-adaptation induced thermotolerance in *Staphylococcus aureus*: Influence of the alternative factor σ^B', *International Journal of Food Microbiology*, vol. 135, no. 3, pp. 274-280.

Chae, MS, Schraft, H, Hansen, LT & Mackereth, R 2006, 'Effects of physicochemical surface characteristics of Listeria monocytogenes strains on attachment to glass', *Food Microbiology*, vol. 23, no. 3, pp. 250-259.

Champomier-Vergès, M-C, Maguin, E, Mistou, M-Y, Anglade, P & Chich, J-F 2002, 'Lactic acid bacteria and proteomics: current knowledge and perspectives', *Journal of Chromatography B*, vol. 771, no. 1, pp. 329-342.

Chandry, P, Moore, S, Davidson, B & Hillier, A 1998, 'Investigation of the microbial ecology of maturing cheese by PCR and PFGE', *Australian Journal of Dairy Technology*, vol. 53, no. 2, p. 117.

Chandry, P, Moore, S & Hillier, A 2002, 'Microecology of NSLAB in Australian cheddar', *Australian Journal of Dairy Technology*, vol. 57, no. 2, p. 106.

Chao, MC, Kieser, KJ, Minami, S, Mavrici, D, Aldridge, BB, Fortune, SM, Alber, T & Rubin, EJ 2013, 'Protein complexes and proteolytic activation of the cell wall hydrolase RipA regulate septal resolution in mycobacteria', *PLoS Pathogens*, vol. 9, no. 2, p. e1003197.

Chao, S-H, Tomii, Y, Sasamoto, M, Fujimoto, J, Tsai, Y-C & Watanabe, K 2008, '*Lactobacillus capillatus* sp. nov., a motile bacterium isolated from stinky tofu brine', *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 11, pp. 2555-2559.

Chapot-Chartier, M-P 2014, 'Interactions of the cell-wall glycopolymers of lactic acid bacteria with their bacteriophages', *Frontiers in Microbiology*, vol. 5.

Chapot-Chartier, M-P & Kulakauskas, S 2014, 'Cell wall structure and function in lactic acid bacteria', in *Microbial Cell Factories*, vol. 13, p. S9.

Chastanet, A, Fert, J & Msadek, T 2003, 'Comparative genomics reveal novel heat shock regulatory mechanisms in *Staphylococcus aureus* and other Gram-positive bacteria', *Molecular Microbiology*, vol. 47, no. 4, pp. 1061-1073.

Chastanet, A & Msadek, T 2003, 'ClpP of *Streptococcus salivarius* is a novel member of the dually regulated class of stress response genes in Gram-positive bacteria', *Journal of Bacteriology*, vol. 185, no. 2, pp. 683-687.

Chatfield, CH, Koo, H & Quivey Jr, RG 2005, 'The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated', *Microbiology*, vol. 151, no. 2, pp. 625-631.

Chaturongakul, S & Boor, KJ 2006, 'σ^B activation under environmental and energy stress conditions in *Listeria monocytogenes'*, *Applied and Environmental Microbiology*, vol. 72, no. 8, pp. 5197-5203.

Chatzi, KE, Sardis, MF, Karamanou, S & Economou, A 2013, 'Breaking on through to the other side: protein export through the bacterial Sec system', *Biochemical Journal*, vol. 449, no. 1, pp. 25-37.

Chávez-Tapia, NC, González-Rodríguez, L, Jeong, M, López-Ramírez, Y, Barbero-Becerra, V, Juárez-Hernández, E, Romero-Flores, JL, Arrese, M, Méndez-Sánchez, N & Uribe, M 2015, 'Current evidence on the use of probiotics in liver diseases', *Journal of Functional Foods*, vol. 17, pp. 137-151.

Chen, H, Chen, S, Li, C & Shu, G 2015, 'Response surface optimization of lyoprotectant for *Lactobacillus bulgaricus* during vacuum freeze-drying', *Preparative Biochemistry and Biotechnology*, vol. 45, no. 5, pp. 463-475.

Chen, X, Xu, J, Shuai, J, Chen, J, Zhang, Z & Fang, W 2007, 'The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157: H7 and *Salmonella typhimurium*', *International Journal of Food Microbiology*, vol. 115, no. 3, pp. 307-312.

Chou, K-C & Shen, H-B 2010, 'Cell-PLoc 2.0: An improved package of web-servers for predicting subcellular localization of proteins in various organisms', *Natural Science*, vol. 2, no. 10, p. 1090.

Coeuret, V, Gueguen, M & Vernoux, JP 2004, 'Numbers and strains of lactobacilli in some probiotic products', *International Journal of Food Microbiology*, vol. 97, no. 2, pp. 147-156.

Cohen, D, Renes, J, Bouwman, FG, Zoetendal, EG, Mariman, E, de Vos, WM & Vaughan, EE 2006, 'Proteomic analysis of log to stationary growth phase *Lactobacillus plantarum* cells and a 2-DE database', *Proteomics*, vol. 6, no. 24, pp. 6485-6493.

Condon, S 1987, 'Responses of lactic acid bacteria to oxygen', *Fems Microbiology Letters*, vol. 46, no. 3, pp. 269-280.

UniProt Consortium, 2016, 'UniProt: the universal protein knowledgebase', *Nucleic Acids Research*, vol. 45, no. D1, pp. D158-D169.

Coordinators, NR 2017, 'Database resources of the national center for biotechnology information', *Nucleic Acids Research*, vol. 45, no. Database issue, p. D12.

Corcoran, B, Stanton, C, Fitzgerald, G & Ross, R 2008, 'Life under stress: the probiotic stress response and how it may be manipulated', *Current Pharmaceutical Design*, vol. 14, no. 14, pp. 1382-1399.

Cotter, PD & Hill, C 2003, 'Surviving the acid test: responses of Gram-positive bacteria to low pH', *Microbiology and Molecular Biology Reviews*, vol. 67, no. 3, pp. 429-453.

Cox, J, Hein, MY, Luber, CA, Paron, I, Nagaraj, N & Mann, M 2014, 'Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ', *Molecular & Cellular Proteomics*, vol. 13, no. 9, pp. 2513-2526.

Cox, J, Neuhauser, N, Michalski, A, Scheltema, RA, Olsen, JV & Mann, M 2011, 'Andromeda: a peptide search engine integrated into the MaxQuant environment', *Journal of Proteome Research*, vol. 10, no. 4, pp. 1794-1805.

Creecy, JP & Conway, T 2015, 'Quantitative bacterial transcriptomics with RNA-seq', *Current Opinion in Microbiology*, vol. 23, pp. 133-140.

De Angelis, M, Bini, L, Pallini, V, Cocconcelli, PS & Gobbetti, M 2001, 'The acid-stress response in *Lactobacillus sanfranciscensis* CB1', *Microbiology*, vol. 147, no. 7, pp. 1863-1873.

De Angelis, M, Calasso, M, Cavallo, N, Di Cagno, R & Gobbetti, M 2016, 'Functional proteomics within the genus *Lactobacillus*', *Proteomics*, vol.16, no. 6, pp. 946-62.

De Angelis, M, Corsetti, A, Tosti, N, Rossi, J, Corbo, M & Gobbetti, M 2001, 'Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analyses', *Applied and environmental microbiology*, vol. 67, no. 5, pp. 2011-2020.

De Angelis, M, Di Cagno, R, Huet, C, Crecchio, C, Fox, PF & Gobbetti, M 2004, 'Heat shock response in *Lactobacillus plantarum*', *Applied and Environmental Microbiology*, vol. 70, no. 3, pp. 1336-1346.

De Angelis, M & Gobbetti, M 2004, 'Environmental stress responses in *Lactobacillus*: a review', *Proteomics*, vol. 4, no. 1, pp. 106-122.

De Angelis, Maria, and Marco Gobbetti, 2011, 'Stress responses of lactobacilli', in *Stress responses of Lactic Acid Bacteria*, Springer, pp. 219-249.

De Angelis, M, Mariotti, L, Rossi, J, Servili, M, Fox, PF, Rollán, G & Gobbetti, M 2002, 'Arginine catabolism by sourdough lactic acid bacteria: purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1', *Applied and Environmental Microbiology*, vol. 68, no. 12, pp. 6193-6201.

de Figueiredo, HM, Passos, FJV, Passos, FML, Lourenço, LdFH, Araujo, EAF, da Silva, LHM & de Moura Guimarães, PC 2015, 'Powdered milk containing *Lactobacillus acidophilus* isolated in Brazil', *Journal of Food Science and Technology*, vol. 52, no. 1, pp. 562-567.

de Gooijer, CD, Bakker, WA, Beeftink, HH & Tramper, J 1996, 'Bioreactors in series: an overview of design procedures and practical applications', *Enzyme and Microbial Technology*, vol. 18, no. 3, pp. 202-219.

De Leblanc, AdM, Matar, C & Perdigón, G 2007, 'The application of probiotics in cancer', *British Journal of Nutrition*, vol. 98, no. S1, pp. S105-S110.

de los Angeles Pineda, M, Thompson, SF, Summers, K, de Leon, F, Pope, J & Reid, G 2011, 'A randomized, double-blinded, placebo-controlled pilot study of probiotics in active rheumatoid arthritis', *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, vol. 17, no. 6, p. CR347.

de Saro, FJL, Yoshikawa, N & Helmann, JD 1999, 'Expression, abundance, and RNA polymerase binding properties of the δ factor of *Bacillus subtilis*', *Journal of Biological Chemistry*, vol. 274, no. 22, pp. 15953-15958.

De Spicer, PO & Maloy, S 1993, 'PutA protein, a membrane-associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator', *Proceedings of the National Academy of Sciences*, vol. 90, no. 9, pp. 4295-4298.

De Vuyst, L & Degeest, B 1999, 'Heteropolysaccharides from lactic acid bacteria', *FEMS Microbiology Reviews*, vol. 23, no. 2, pp. 153-177.

Deepika, G, Green, RJ, Frazier, RA & Charalampopoulos, D 2009, 'Effect of growth time on the surface and adhesion properties of *Lactobacillus rhamnosus* GG', *Journal of Applied Microbiology*, vol. 107, no. 4, pp. 1230-1240.

den Camp, HO, Oosterhof, A & Veerkamp, J 1985, 'Interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells', *Infection and Immunity*, vol. 47, no. 1, pp. 332-334.

Dereeper, A, Audic, S, Claverie, J-M & Blanc, G 2010, 'Blast-Explorer helps you building datasets for phylogenetic analysis', *BMC Evolutionary Biology*, vol. 10, no. 1, p. 8.

Derré, I, Rapoport, G & Msadek, T 1999, 'CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in Gram-positive bacteria', *Molecular Microbiology*, vol. 31, no. 1, pp. 117-131.

Desmond, C, Fitzgerald, G, Stanton, C & Ross, R 2004, 'Improved stress tolerance of GroESL-overproducing *Lactococcus lactis* and probiotic *Lactobacillus paracasei* NFBC 338', *Applied and Environmental Microbiology*, vol. 70, no. 10, pp. 5929-5936.

Desmond, C, Stanton, C, Fitzgerald, GF, Collins, K & Ross, RP 2001, 'Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying', *International Dairy Journal*, vol. 11, no. 10, pp. 801-808.

Di Cagno, R, De Angelis, M, Coda, R, Minervini, F & Gobbetti, M 2009, 'Molecular adaptation of sourdough *Lactobacillus plantarum* DC400 under co-cultivation with other lactobacilli', *Research in Microbiology*, vol. 160, no. 5, pp. 358-366.

Di Cagno, R, De Angelis, M, Limitone, A, Fox, PF & Gobbetti, M 2006, 'Response of *Lactobacillus helveticus* PR4 to heat stress during propagation in cheese whey with a gradient of decreasing temperatures', *Applied and Environmental Microbiology*, vol. 72, no. 7, pp. 4503-4514.

Dionisi, F, Golay, P-A, Elli, M & Fay, LB 1999, 'Stability of cyclopropane and conjugated linoleic acids during fatty acid quantification in lactic acid bacteria', *Lipids*, vol. 34, no. 10, pp. 1107-1115.

Domínguez Rubio, AP, Martínez, JH, Martínez Casillas, DC, Coluccio Leskow, F, Piuri, M & Pérez, OE 2017, '*Lactobacillus casei* BL23 produces microvesicles carrying proteins that have been associated with its probiotic effect', *Frontiers in Microbiology*, vol. 8, p. 1783.

Dubernet, S, Desmasures, N & Guéguen, M 2002, 'A PCR-based method for identification of lactobacilli at the genus level', *FEMS Microbiology Letters*, vol. 214, no. 2, pp. 271-275.

Dubrac, S, Boneca, IG, Poupel, O & Msadek, T 2007, 'New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*', *Journal of Bacteriology*, vol. 189, no. 22, pp. 8257-8269.

Dunne, C 2001, 'Adaptation of bacteria to the intestinal niche: probiotics and gut disorder', *Inflammatory Bowel Diseases*, vol. 7, no. 2, pp. 136-145.

Duong, F & Wickner, W 1997, 'The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling', *The EMBO Journal*, vol. 16, no. 16, pp. 4871-4879.

du Toit, M, Engelbrecht, L, Lerm, E & Krieger-Weber, S 2011, '*Lactobacillus*: the next generation of malolactic fermentation starter cultures—an overview', *Food and Bioprocess Technology*, vol. 4, no. 6, pp. 876-906.

Ebbole, DJ & Zalkin, H 1989, 'Interaction of a putative repressor protein with an extended control region of the *Bacillus subtilis pur* operon', *Journal of Biological Chemistry*, vol. 264, no. 6, pp. 3553-3561.

Edelman, SM, Lehti, TA, Kainulainen, V, Antikainen, J, Kylväjä, R, Baumann, M, Westerlund-Wikström, B & Korhonen, TK 2012, 'Identification of a high-molecular-mass *Lactobacillus* epithelium adhesin (LEA) of *Lactobacillus crispatus* ST1 that binds to stratified squamous epithelium', *Microbiology*, vol. 158, no. 7, pp. 1713-1722.

El Soda, M, Madkor, S & Tong, P 2000, 'Adjunct cultures: recent developments and potential significance to the cheese industry', *Journal of Dairy Science*, vol. 83, no. 4, pp. 609-619.

Elo, S, Saxelin, M & Salminen, S 1991, 'Attachment of *Lactobacillus casei* strain GG to human colon carcinoma cell line Caco-2: comparison with other dairy strains', *Letters in Applied Microbiology*, vol. 13, no. 3, pp. 154-156.

Elsholz, AK, Gerth, U & Hecker, M 2010, 'Regulation of CtsR activity in low GC, Gram+ bacteria', *Advances in Microbial Physiology*, vol 57, pp. 119-144.
Emanuelsson, O, Nielsen, H, Brunak, S & Von Heijne, G 2000, 'Predicting subcellular localization of proteins based on their N-terminal amino acid sequence', *Journal of Molecular Biology*, vol. 300, no. 4, pp. 1005-1016.

Espino, E, Koskenniemi, K, Mato-Rodriguez, L, Nyman, TA, Reunanen, J, Koponen, J, Öhman, T, Siljamäki, P, Alatossava, T & Varmanen, P 2014, 'Uncovering surface-exposed antigens of *Lactobacillus rhamnosus* by cell shaving proteomics and two-dimensional immunoblotting', *Journal of Proteome Research*, vol. 14, no. 2, pp. 1010-1024.

Espino, E, Koskenniemi, K, Mato-Rodriguez, L, Nyman, TA, Reunanen, J, Koponen, J, Ohman, T, Siljamaki, P, Alatossava, T, Varmanen, P & Savijoki, K 2015, 'Uncovering Surface-Exposed Antigens of *Lactobacillus rhamnosus* by Cell Shaving Proteomics and Two-Dimensional Immunoblotting', *Journal of Proteome Research*, vol. 14, no. 2, pp. 1010-1024.

Etzold, S, Kober, OI, MacKenzie, DA, Tailford, LE, Gunning, AP, Walshaw, J, Hemmings, AM & Juge, N 2014, 'Structural basis for adaptation of lactobacilli to gastrointestinal mucus', *Environmental Microbiology*, vol. 16, no. 3, pp. 888-903.

Even, S, Lindley, ND, Loubière, P & Cocaign-Bousquet, M 2002, 'Dynamic response of catabolic pathways to autoacidification in *Lactococcus lactis*: transcript profiling and stability in relation to metabolic and energetic constraints', *Molecular Microbiology*, vol. 45, no. 4, pp. 1143-1152.

Fanning, S, Hall, LJ, Cronin, M, Zomer, A, MacSharry, J, Goulding, D, Motherway, MOC, Shanahan, F, Nally, K & Dougan, G 2012, 'Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection', *Proceedings of the National Academy of Sciences*, vol. 109, no. 6, pp. 2108-2113.

FDA, 2010, 'Generally Recognized as Safe (GRAS) Notifications', https://www.fda.gov/AnimalVeterinary/Products/AnimalFoodFeeds/GenerallyRecognizedasSafeGRASNotifications/default.htm

Fernandez, A, Ogawa, J, Penaud, S, Boudebbouze, S, Ehrlich, D, van de Guchte, M & Maguin, E 2008, 'Rerouting of pyruvate metabolism during acid adaptation in *Lactobacillus bulgaricus*', *Proteomics*, vol. 8, no. 15, pp. 3154-3163.

Ferrando, V, Quiberoni, A, Reinhemer, J & Suárez, V 2015, 'Resistance of functional *Lactobacillus plantarum* strains against food stress conditions', *Food Microbiology*, vol. 48, pp. 63-71.

Fiocco, D, Capozzi, V, Collins, M, Gallone, A, Hols, P, Guzzo, J, Weidmann, S, Rieu, A, Msadek, T & Spano, G 2010, 'Characterization of the CtsR stress response regulon in *Lactobacillus plantarum*', *Journal of Bacteriology*, vol. 192, no. 3, pp. 896-900.

Firuzi, O, Miri, R, Tavakkoli, M & Saso, L 2011, 'Antioxidant therapy: current status and future prospects', *Current Medicinal Chemistry*, vol. 18, no. 25, pp. 3871-3888.

Flora, S 2006, 'Role of free radicals and antioxidants in health and disease', *Cellular and Molecular Biology (Noisy-le-Grand, France)*, vol. 53, no. 1, pp. 1-2.

Food, Organization, A & Organization, WH 2006, *Probiotics in food: health and nutritional properties and guidelines for evaluation*, FAO.

Foschi, C, Laghi, L, Parolin, C, Giordani, B, Compri, M, Cevenini, R, Marangoni, A & Vitali, B 2017, 'Novel approaches for the taxonomic and metabolic characterization of lactobacilli: Integration of 16S rRNA gene sequencing with MALDI-TOF MS and 1H-NMR', *PloS One*, vol. 12, no. 2, p. e0172483.

Fox, P, McSweeney, P & Lynch, C 1998, 'Significance of non-starter lactic acid bacteria in Chedder cheese', *Australian Journal of Dairy Technology*, vol. 53, no. 2, p. 83.

Fox, PF & McSweeney, PL 2017, 'Cheese: an overview', in *Cheese (Fourth Edition)*, Elsevier, pp. 5-21.

Francke, C, Kormelink, TG, Hagemeijer, Y, Overmars, L, Sluijter, V, Moezelaar, R & Siezen, RJ 2011, 'Comparative analyses imply that the enigmatic Sigma factor 54 is a central controller of the bacterial exterior', *BMC Genomics*, vol. 12, no. 1, p. 385.

Fraqueza, MJ 2015, 'Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages', *International Journal of Food Microbiology*, vol. 212, pp. 76-88.

Fraser, CM, Gocayne, JD, White, O & Adams, MD 1995, 'The minimal gene complement of *Mycoplasma genitalium*', *Science*, vol. 270, no. 5235, p. 197.

Frece, J, Kos, B, Svetec, I-K, Zgaga, Z, Mrša, V & Šušković, J 2005, 'Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92', *Journal of Applied Microbiology*, vol. 98, no. 2, pp. 285-292.

Frees, D, Savijoki, K, Varmanen, P & Ingmer, H 2007, 'Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria', *Molecular Microbiology*, vol. 63, no. 5, pp. 1285-1295.

Fuller, R 1991, 'Probiotics in human medicine', *Gut*, vol. 32, no. 4, p. 439.

Ganesan, B, Weimer, B, Pinzon, J, Dao Kong, N, Rompato, G, Brothersen, C & McMahon, D 2014, 'Probiotic bacteria survive in Cheddar cheese and modify populations of other lactic acid bacteria', *Journal of Applied Microbiology*, vol. 116, no. 6, pp. 1642-1656.

Garnier, M, Matamoros, S, Chevret, D, Pilet, M-F, Leroi, F & Tresse, O 2010, 'Adaptation to cold and proteomic responses of the psychrotrophic biopreservative *Lactococcus piscium* strain CNCM I-4031', *Applied and Environmental Microbiology*, vol. 76, no. 24, pp. 8011-8018.

Garrity, GM, Bell, JA & Lilburn, T 2004, 'Taxonomic outline of the prokaryotes. Bergey's manual of systematic *Bacteriology*', *Springer, New York, Berlin, Heidelberg*.

Garrote, GL, Delfederico, L, Bibiloni, R, Abraham, AG, Pérez, PF, Semorile, L & De Antoni, GL 2004, 'Lactobacilli isolated from kefir grains: evidence of the presence of S-layer proteins', *Journal of Dairy Research*, vol. 71, no. 2, pp. 222-230.

Georgieva, RP, Koleva, D, Nikolova, D, Yankov, and Danova S 2009, 'Growth parameters of probiotic strain *Lactobacillus plantarum*, isolated from traditional white cheese', *Biotechnology & Biotechnological Equipment*, vol. 23, no. sup1, pp. 861-865.

Giraffa, G, Chanishvili, N & Widyastuti, Y 2010, 'Importance of lactobacilli in food and feed biotechnology', *Research in Microbiology*, vol. 161, no. 6, pp. 480-487.

Givskov, M, Eberl, L, Møller, S, Poulsen, LK & Molin, S 1994, 'Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content', *Journal of Bacteriology*, vol. 176, no. 1, pp. 7-14.

Glaasker, E, Konings, WN & Poolman, B 1996a, 'Glycine betaine fluxes in *Lactobacillus plantarum* during osmostasis and hyper-and hypo-osmotic shock', *Journal of Biological Chemistry*, vol. 271, no. 17, pp. 10060-10065.

Glaasker, E, Konings, WN & Poolman, B 1996b, 'Osmotic regulation of intracellular solute pools in *Lactobacillus plantarum*', *Journal of Bacteriology*, vol. 178, no. 3, pp. 575-582.

Glenting, J, Beck, HC, Vrang, A, Riemann, H, Ravn, P, Hansen, AM, Antonsson, M, Ahrné, S, Israelsen, H & Madsen, S 2013, 'Anchorless surface associated glycolytic enzymes from *Lactobacillus plantarum* 299v bind to epithelial cells and extracellular matrix proteins', *Microbiological Research*, vol. 168, no. 5, pp. 245-253.

Gobbetti, M, De Angelis, M, Corsetti, A & Di Cagno, R 2005, 'Biochemistry and physiology of sourdough lactic acid bacteria', *Trends in Food Science & Technology*, vol. 16, no. 1-3, pp. 57-69.

Gogineni, VK, Morrow, LE & Malesker, MA 2013, 'Probiotics: mechanisms of action and clinical applications', *Journal of Probiotic Health*, vol. 1, no. 101, p. 2.

Goldstein, EJ, Tyrrell, KL & Citron, DM 2015, '*Lactobacillus* species: taxonomic complexity and controversial susceptibilities', *Clinical Infectious Diseases*, vol. 60, no. suppl_2, pp. S98-S107.

González-Rodríguez, I, Sánchez, B, Ruiz, L, Turroni, F, Ventura, M, Ruas-Madiedo, P, Gueimonde, M & Margolles, A 2012, 'Role of extracellular transaldolase from *Bifidobacterium bifidum* in mucin adhesion and aggregation', *Applied and Environmental Microbiology*, vol. 78, no. 11, pp. 3992-3998.

Gorg, A, Weiss, W & Dunn, MJ 2004, 'Current two-dimensional electrophoresis technology for proteomics', *Proteomics*, vol. 4, no. 12, pp. 3665-3685.

Gotoh, Y, Doi, A, Furuta, E, Dubrac, S, Ishizaki, Y, Okada, M, Igarashi, M, Misawa, N, Yoshikawa, H & Okajima, T 2010, 'Novel antibacterial compounds specifically targeting the essential WalR response regulator', *The Journal of Antibiotics*, vol. 63, no. 3, p. 127.

Gottesman, S, Wickner, S, Maurizi, MR, Beals, CR, Clipstone, NA, Ho, SN, Crabtree, GR, Zamir, I, Zhang, J & Lazar, MA 1997, 'Protein quality control: triage by chaperones and proteases 815', *Genes Dev*, vol. 11, pp. 815-823.

Govender, M, Choonara, YE, Kumar, P, du Toit, LC, van Vuuren, S & Pillay, V 2014, 'A review of the advancements in probiotic delivery: Conventional vs. non-conventional formulations for intestinal flora supplementation', *Aaps PharmSciTech*, vol. 15, no. 1, pp. 29-43.

Granato, D, Perotti, F, Masserey, I, Rouvet, M, Golliard, M, Servin, A & Brassart, D 1999, 'Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells', *Applied and Environmental Microbiology*, vol. 65, no. 3, pp. 1071-1077.

Grandvalet, C, Coucheney, F, Beltramo, C & Guzzo, J 2005, 'CtsR is the master regulator of stress response gene expression in *Oenococcus oeni*', *Journal of Bacteriology*, vol. 187, no. 16, pp. 5614-5623.

Grandvalet, C, Servant, P & Mazodier, P 1997, 'Disruption of *hspR*, the repressor gene of the *dnaK* operon in *Streptomyces albus* G', *Molecular Microbiology*, vol. 23, no. 1, pp. 77-84.

Greene, JD & Klaenhammer, TR 1994, 'Factors involved in adherence of lactobacilli to human Caco-2 cells', *Applied and Environmental Microbiology*, vol. 60, no. 12, pp. 4487-4494.

Grossman, AD, Straus, DB, Walter, WA & Gross, CA 1987, 'Sigma 32 synthesis can regulate the synthesis of heat shock proteins in *Escherichia coli*', *Genes and Development*, vol. 1, no. 2, pp. 179-184.

Guerzoni, ME, Lanciotti, R & Cocconcelli, PS 2001, 'Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*', *Microbiology*, vol. 147, no. 8, pp. 2255-2264.

Hahne, H, Mäder, U, Otto, A, Bonn, F, Steil, L, Bremer, E, Hecker, M & Becher, D 2010, 'A comprehensive proteomics and transcriptomics analysis of *Bacillus subtilis* salt stress adaptation', *Journal of Bacteriology*, vol. 192, no. 3, pp. 870-882.

Hamed, RB, Batchelar, ET, Clifton, I & Schofield, CJ 2008, 'Mechanisms and structures of crotonase superfamily enzymes–how nature controls enolate and oxyanion reactivity', *Cellular and Molecular Life Sciences*, vol. 65, no. 16, pp. 2507-2527.

Hammerschmidt, S, Agarwal, V, Kunert, A, Haelbich, S, Skerka, C & Zipfel, PF 2007, 'The host immune regulator factor H interacts via two contact sites with the PspC protein of *Streptococcus pneumoniae* and mediates adhesion to host epithelial cells', *The Journal of Immunology*, vol. 178, no. 9, pp. 5848-5858.

Hammes, F, Berney, M & Egli, T 2010, 'Cultivation-independent assessment of bacterial viability', in *High resolution microbial single cell analytics*, Springer, pp. 123-150.

Hannavy, K, Rospert, S & Schatz, G 1993, 'Protein import into mitochondria: a paradigm for the translocation of polypeptides across membranes', *Current opinion in cell biology*, vol. 5, no. 4, pp. 694-700.

Hartl, FU 1996, 'Molecular chaperones in cellular protein folding', *Nature*, vol. 381, no. 6583, p. 571.

Hatakka, K, Martio, J, Korpela, M, Herranen, M, Poussa, T, Laasanen, T, Saxelin, M, Vapaatalo, H, Moilanen, E & Korpela, R 2003, 'Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis - a pilot study', *Scandinavian Journal of Rheumatology*, vol. 32, no. 4, pp. 211-215.

Haydon, DJ & Guest, JR 1991, 'A new family of bacterial regulatory proteins', *FEMS Microbiology Letters*, vol. 79, no. 2-3, pp. 291-296.

Heeney, DD, Gareau, MG & Marco, ML 2018, 'Intestinal *Lactobacillus* in health and disease, a driver or just along for the ride? *Current Opinion in Biotechnology*, vol. 49, pp. 140-147.

Heinemann, C, van Hylckama Vlieg, JE, Janssen, DB, Busscher, HJ, van der Mei, HC & Reid, G 2000, 'Purification and characterization of a surface-binding protein from

Lactobacillus fermentum RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131', *Fems Microbiology Letters*, vol. 190, no. 1, pp. 177-180.

Held, WA & Nomura, M 1973, 'Structure and function of bacterial ribosomes. XX. Rate-determining step in the reconstitution of *Escherichia coli* 30S ribosomal subunits', *Biochemistry*, vol. 12, no. 17, pp. 3273-3281.

Heller, KJ 2001, 'Probiotic bacteria in fermented foods: product characteristics and starter organisms', *The American Journal of Clinical Nutrition*, vol. 73, no. 2, pp. 374s-379s.

Heunis, T, Deane, S, Smit, S & Dicks, LMT 2014, 'Proteomic profiling of the acid stress response in *Lactobacillus plantarum* 423', *Journal of Proteome Research*, vol. 13, no. 9, pp. 4028-4039.

Hickey, C, Auty, M, Wilkinson, M & Sheehan, J 2017, 'Influence of process temperature and salting methods on starter and NSLAB growth and enzymatic activity during the ripening of cheeses produced with *Streptococcus thermophilus* and *Lactobacillus helveticus*', *International Dairy Journal*, vol. 69, pp. 9-18.

Hill, C, Guarner, F, Reid, G, Gibson, GR, Merenstein, DJ, Pot, B, Morelli, L, Canani, RB, Flint, HJ & Salminen, S 2014, 'Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic', *Nature Reviews Gastroenterology and Hepatology*, vol. 11, no. 8, p. 506.

Hobman, JL, Wilkie, J & Brown, NL 2005, 'A design for life: prokaryotic metal-binding MerR family regulators', *Biometals*, vol. 18, no. 4, pp. 429-436.

Hochwind, K, Weinmaier, T, Schmid, M, van Hemert, S, Hartmann, A, Rattei, T & Rothballer, M 2012, 'Draft genome sequence of *Lactobacillus casei* W56', *Journal of Bacteriology*, vol. 194, no. 23, pp. 6638-6638.

Hoehn, K & Marieb, EN 2010, *Human anatomy and physiology*, Benjamin Cummings, San Francisco, Calif, USA.

Hörmann, S, Scheyhing, C, Behr, J, Pavlovic, M, Ehrmann, M & Vogel, RF 2006, 'Comparative proteome approach to characterize the high-pressure stress response of *Lactobacillus sanfranciscensis* DSM 20451T', *Proteomics*, vol. 6, no. 6, pp. 1878-1885.

Huang, C, Rossi, P, Saio, T & Kalodimos, CG 2016, 'Structural basis for the antifolding activity of a molecular chaperone', *Nature*, vol. 537, no. 7619, p. 202.

Huberts, DH & van der Klei, IJ 2010, 'Moonlighting proteins: an intriguing mode of multitasking', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, vol. 1803, no. 4, pp. 520-525.

Hurmalainen, V, Edelman, S, Antikainen, J, Baumann, M, Lähteenmäki, K & Korhonen, TK 2007, 'Extracellular proteins of *Lactobacillus crispatus* enhance activation of human plasminogen', *Microbiology*, vol. 153, no. 4, pp. 1112-1122.

Hussain, M, Knight, M & Britz, M 2009, 'Proteomic analysis of lactose-starved *Lactobacillus casei* during stationary growth phase', *Journal of Applied Microbiology*, vol. 106, no. 3, pp. 764-773.

Hussain, M, Peters, G, Chhatwal, GS & Herrmann, M 1999, 'A lithium chloride-extracted, broad-spectrum-adhesive 42-kilodalton protein of *Staphylococcus epidermidis* is ornithine carbamoyltransferase', *Infection and immunity*, vol. 67, no. 12, pp. 6688-6690.

Hussain, MA, Knight, MI & Britz, ML 2013, 'Understanding the starvation adaptation of *Lactobacillus casei* through Proteomics', *Asian Journal of Agriculture and Food Sciences*, vol. 1, no. 5.

Hutkins, RW & Nannen, NL 1993, 'pH homeostasis in lactic acid bacteria', *Journal of Dairy Science*, vol. 76, no. 8, pp. 2354-2365.

Hynönen, U & Palva, A 2013, '*Lactobacillus* surface layer proteins: structure, function and applications', *Applied Microbiology and Biotechnology*, vol. 97, no. 12, pp. 5225-5243.

Ibarburu, I, Puertas, AI, Berregi, I, Rodríguez-Carvajal, MA, Prieto, A & Dueñas, MT 2015, 'Production and partial characterization of exopolysaccharides produced by two *Lactobacillus suebicus* strains isolated from cider', *International Journal of Food Microbiology*, vol. 214, pp. 54-62.

Iyer, R, Baliga, NS & Camilli, A 2005, 'Catabolite control protein A (CcpA) contributes to virulence and regulation of sugar metabolism in *Streptococcus pneumoniae*', *Journal of bacteriology*, vol. 187, no. 24, pp. 8340-8349.

Jeffery, C, Mani, M, Amblee, V & Chen, C 2014, 'Moonlighting Proteins', *Biophysical Journal*, vol. 106, no. 2, pp. 658A-658A.

Jeffery, C, Mani, M, Zabad, S, Amblee, V, Chen, C, Liu, H, Zwicke, G, Mathur, T, Patel, B & Thakkar, J 2015, 'MoonProt: A database for proteins that are known to moonlight', *The FASEB Journal*, vol. 29, no. 1 Supplement, p. 567.510.

Jeffery, CJ 1999, 'Moonlighting proteins', *Trends in biochemical sciences*, vol. 24, no. 1, pp. 8-11.

Jeffery, CJ 2009, 'Moonlighting proteins - an update', *Molecular BioSystems*, vol. 5, no. 4, pp. 345-350.

Jeffery, CJ 2014, 'An introduction to protein moonlighting', *Biochemical Society Transactions*, vol. 42, pp. 1679-1683.

Jerga, A & Rock, CO 2009, 'Acyl-acyl carrier protein regulates transcription of fatty acid biosynthetic genes via the FabT repressor in *Streptococcus pneumoniae*', *Journal of Biological Chemistry*, vol. 284, no. 23, pp. 15364-15368.

Jitrapakdee, S & Wallace, JC 2003, 'The biotin enzyme family: conserved structural motifs and domain rearrangements', *Current Protein and Peptide Science*, vol. 4, no. 3, pp. 217-229.

Jobin, MP, Delmas, F, Garmyn, D, Divies, C & Guzzo, J 1998, 'Characterization of small heat shock proteins in lactic acid bacteria', *Le Lait*, vol. 1, no 78, pp. 165-71.

Johnson, B, Selle, K, O'Flaherty, S, Goh, YJ & Klaenhammer, T 2013, 'Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM', *Microbiology*, vol. 159, no. Pt 11, pp. 2269-2282.

Johnson, BR, Hymes, J, Sanozky-Dawes, R, Henriksen, ED, Barrangou, R & Klaenhammer, TR 2016, 'Conserved S-layer-associated proteins revealed by exoproteomic survey of S-layer-forming lactobacilli', *Applied and Environmental Microbiology*, vol. 82, no. 1, pp. 134-145.

Johnson, JW, Fisher, JF & Mobashery, S 2013, 'Bacterial cell-wall recycling', *Annals of the New York Academy of Sciences*, vol. 1277, no. 1, pp. 54-75.

Jordan, S, Hutchings, MI & Mascher, T 2008, 'Cell envelope stress response in Gram-positive bacteria', *FEMS Microbiology Reviews*, vol. 32, no. 1, pp. 107-146.

Kainulainen, V & Korhonen, TK 2014, 'Dancing to another tune - adhesive moonlighting proteins in bacteria', *Biology*, vol. 3, no. 1, pp. 178-204.

Kainulainen, V, Loimaranta, V, Pekkala, A, Edelman, S, Antikainen, J, Kylväjä, R, Laaksonen, M, Laakkonen, L, Finne, J & Korhonen, TK 2012, 'Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37', *Journal of Bacteriology*, vol. 194, no. 10, pp. 2509-2519.

Kanehisa, M & Goto, S 2000, 'KEGG: kyoto encyclopedia of genes and genomes', *Nucleic Acids Research*, vol. 28, no. 1, pp. 27-30.

Kapila, S & Sinha, P 2006, 'Antioxidative and hypocholesterolemic effect of *Lactobacillus casei* ssp *casei* (biodefensive properties of lactobacilli)', *Indian Journal of Medical Sciences*, vol. 60, no. 9, p. 361.

Kararli, TT 1995, 'Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals', *Biopharmaceutics & Drug Disposition*, vol. 16, no. 5, pp. 351-380.

Kashket, ER 1987, 'Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance', *Fems Microbiology Letters*, vol. 46, no. 3, pp. 233-244.

Kawai, Y, Marles-Wright, J, Cleverley, RM, Emmins, R, Ishikawa, S, Kuwano, M, Heinz, N, Bui, NK, Hoyland, CN & Ogasawara, N 2011, 'A widespread family of bacterial cell wall assembly proteins', *The EMBO Journal*, vol. 30, no. 24, pp. 4931-4941.

Kawai, Y, Moriya, S & Ogasawara, N 2003, 'Identification of a protein, YneA, responsible for cell division suppression during the SOS response in *Bacillus subtilis*', *Molecular Microbiology*, vol. 47, no. 4, pp. 1113-1122.

Kazmierczak, MJ, Wiedmann, M & Boor, KJ 2005, 'Alternative sigma factors and their roles in bacterial virulence', *Microbiology and Molecular Biology Reviews*, vol. 69, no. 4, pp. 527-543.

Kelley, LA, Mezulis, S, Yates, CM, Wass, MN & Sternberg, MJ 2015, 'The Phyre2 web portal for protein modeling, prediction and analysis', *Nature Protocols*, vol. 10, no. 6, p. 845.

Kelly, P, Maguire, PB, Bennett, M, Fitzgerald, DJ, Edwards, RJ, Thiede, B, Treumann, A, Collins, JK, O'sullivan, GC & Shanahan, F 2005, 'Correlation of probiotic *Lactobacillus salivarius* growth phase with its cell wall-associated proteome', *FEMS Microbiology Letters*, vol. 252, no. 1, pp. 153-159.

Kempf, B & Bremer, E 1998, 'Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments', *Archives of Microbiology*, vol. 170, no. 5, pp. 319-330.

Kenney, LJ 2002, 'Structure/function relationships in OmpR and other winged-helix transcription factors', *Current Opinion in Microbiology*, vol. 5, no. 2, pp. 135-141.

Kilstrup, M, Hammer, K, Ruhdal Jensen, P & Martinussen, J 2005, 'Nucleotide metabolism and its control in lactic acid bacteria', *FEMS Microbiology Reviews*, vol. 29, no. 3, pp. 555-590.

Kilstrup, M, Jacobsen, S, Hammer, K & Vogensen, FK 1997, 'Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*', *Applied and Environmental Microbiology*, vol. 63, no. 5, pp. 1826-1837.

Kleerebezem, M, Hols, P, Bernard, E, Rolain, T, Zhou, M, Siezen, RJ & Bron, PA 2010, 'The extracellular biology of the lactobacilli', *FEMS Microbiology Reviews*, vol. 34, no. 2, pp. 199-230.

Klein, AH, Shulla, A, Reimann, SA, Keating, DH & Wolfe, AJ 2007, 'The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators', *Journal of Bacteriology*, vol. 189, no. 15, pp. 5574-5581.

Klein, G, Pack, A, Bonaparte, C & Reuter, G 1998, 'Taxonomy and physiology of probiotic lactic acid bacteria', *International Journal of Food Microbiology*, vol. 41, no. 2, pp. 103-125.

Klotz, C, O'Flaherty, S, Goh, YJ & Barrangou, R 2017, 'Investigating the Effect of Growth Phase on the Surface-Layer Associated Proteome of *Lactobacillus acidophilus* Using Quantitative Proteomics', *Frontiers in Microbiology*, vol. 8, p. 2174.

Kocharunchitt, C, King, T, Gobius, K, Bowman, JP & Ross, T 2012, 'Integrated transcriptomic and proteomic analysis of the physiological response of *Escherichia coli* O157: H7 Sakai to steady-state conditions of cold and water activity stress', *Molecular & Cellular Proteomics*, vol. 11, no. 1, p. M111. 009019.

Köller, T, Nelson, D, Nakata, M, Kreutzer, M, Fischetti, VA, Glocker, MO, Podbielski, A & Kreikemeyer, B 2008, 'PlyC, a novel bacteriophage lysin for compartment-dependent proteomics of group A streptococci', *Proteomics*, vol. 8, no. 1, pp. 140-148.

Konings, W, Lolkema, J, Bolhuis, H, Van Veen, H, Poolman, B & Driessen, A 1997, 'The role of transport processes in survival of lactic acid bacteria, Energy transduction and multidrug resistance', *Antonie Van Leeuwenhoek*, vol. 71, no. 1, pp. 117-128.

Konings, WN 2002, 'The cell membrane and the struggle for life of lactic acid bacteria', in *Lactic Acid Bacteria: Genetics, Metabolism and Applications*, Springer, pp. 3-27.

Koponen, J, Laakso, K, Koskenniemi, K, Kankainen, M, Savijoki, K, Nyman, TA, de Vos, WM, Tynkkynen, S, Kalkkinen, N & Varmanen, P 2012, 'Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG', *Journal of Proteomics*, vol. 75, no. 4, pp. 1357-1374.

Kormelink, TG, Koenders, E, Hagemeijer, Y, Overmars, L, Siezen, RJ, de Vos, WM & Francke, C 2012, 'Comparative genome analysis of central nitrogen metabolism and its control by GlnR in the class Bacilli', *BMC Genomics*, vol. 13, no. 1, p. 191.

Kourkoutas, Y, Bosnea, L, Taboukos, S, Baras, C, Lambrou, D & Kanellaki, M 2006, 'Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces', *Journal of Dairy Science*, vol. 89, no. 5, pp. 1439-1451.

Kowalska, E & Kozik, A 2008, 'The genes and enzymes involved in the biosynthesis of thiamin and thiamin diphosphate in yeasts', *Cellular & Molecular Biology Letters*, vol. 13, no. 2, p. 271.

Krogh, A, Larsson, B, Von Heijne, G & Sonnhammer, EL 2001, 'Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes', *Journal of Molecular Biology*, vol. 305, no. 3, pp. 567-580.

Krüger, E, Zühlke, D, Witt, E, Ludwig, H & Hecker, M 2001, 'Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor', *The EMBO Journal*, vol. 20, no. 4, pp. 852-863.

Kruger, NJ 1994, 'The Bradford method for protein quantitation', *Basic Protein and Peptide Protocols*, pp. 9-15.

Kruger, NJ 2002, 'The Bradford method for protein quantitation', in *The Protein Protocols Handbook*, Springer, pp. 15-21.

Kumamoto, CA 1989, '*Escherichia coli* SecB protein associates with exported protein precursors in vivo', *Proceedings of the National Academy of Sciences*, vol. 86, no. 14, pp. 5320-5324.

Kumar, M, Kumar, A, Nagpal, R, Mohania, D, Behare, P, Verma, V, Kumar, P, Poddar, D, Aggarwal, P & Henry, C 2010, 'Cancer-preventing attributes of probiotics: an update', *International Journal of Food Sciences and Nutrition*, vol. 61, no. 5, pp. 473-496.

Kumar, M, Nagpal, R, Kumar, R, Hemalatha, R, Verma, V, Kumar, A, Chakraborty, C, Singh, B, Marotta, F & Jain, S 2012, 'Cholesterol-lowering probiotics as potential biotherapeutics for metabolic diseases', *Experimental Diabetes Research*, vol. 2012.

Kunst, F, Ogasawara, N, Moszer, I, Albertini, A, Alloni, G, Azevedo, V, Bertero, M, Bessieres, P, Bolotin, A & Borchert, S 1997, 'The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*', *Nature*, vol. 390, no. 6657, p. 249.

Kuroda, M, Ohta, T, Uchiyama, I, Baba, T, Yuzawa, H, Kobayashi, I, Cui, L, Oguchi, A, Aoki, K-i & Nagai, Y 2001, 'Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*', *The Lancet*, vol. 357, no. 9264, pp. 1225-1240.

Laakso, K, Koskenniemi, K, Koponen, J, Kankainen, M, Surakka, A, Salusjärvi, T, Auvinen, P, Savijoki, K, Nyman, TA & Kalkkinen, N 2011, 'Growth phase-associated changes in the proteome and transcriptome of *Lactobacillus rhamnosus* GG in industrial-type whey medium', *Microbial Biotechnology*, vol. 4, no. 6, pp. 746-766.

Laemmli, U 1970, 'Denaturing (SDS) discontinuous gel electrophoresis', *Nature*, vol. 277, pp. 680-685.

Landete, JM, Ferrer, S, Monedero, V & Zúñiga, M 2013, 'Malic enzyme and malolactic enzyme pathways are functionally linked but independently regulated in *Lactobacillus casei* BL23', *Applied and Environmental Microbiology*, pp.AEM-01177.

Lapsiri, W, Bhandari, B & Wanchaitanawong, P 2013, 'Stability and probiotic properties of *Lactobacillus plantarum* spray-dried with protein and other protectants', *Drying Technology*, vol. 31, no. 13-14, pp. 1723-1733.

Latousakis, D & Juge, N 2018, 'How Sweet Are Our Gut Beneficial Bacteria? A Focus on Protein Glycosylation in *Lactobacillus*', *International Journal of Molecular Sciences*, vol. 19, no. 1, p. 136.

Law, J & Haandrikman, A 1997, 'Proteolytic enzymes of lactic acid bacteria', *International Dairy Journal*, vol. 7, no. 1, pp. 1-11.

Lawson, PA, Citron, DM, Tyrrell, KL & Finegold, SM 2016, 'Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O'Toole 1935) Prévot 1938', *Anaerobe*, vol. 40, pp. 95-99.

Layec, S, Decaris, B & Leblond-Bourget, N 2008, 'Diversity of Firmicutes peptidoglycan hydrolases and specificities of those involved in daughter cell separation', *Research in Microbiology*, vol. 159, no. 7-8, pp. 507-515.

Le Maréchal, C, Peton, V, Plé, C, Vroland, C, Jardin, J, Briard-Bion, V, Durant, G, Chuat, V, Loux, V & Foligné, B 2015, 'Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties', *Journal of Proteomics*, vol. 113, pp. 447-461.

Lebeer, S, Claes, IJ, Balog, CI, Schoofs, G, Verhoeven, TL, Nys, K, von Ossowski, I, de Vos, WM, Tytgat, HL & Agostinis, P 2012, 'The major secreted protein Msp1/p75 is O-glycosylated in *Lactobacillus rhamnosus* GG', *Microbial Cell Factories*, vol. 11, no. 1, p. 15.

Lebeer, S, Vanderleyden, J & De Keersmaecker, SC 2010, 'Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens', *Nature Reviews Microbiology*, vol. 8, no. 3, p. 171.

Lechardeur, D, Cesselin, B, Fernandez, A, Lamberet, G, Garrigues, C, Pedersen, M, Gaudu, P & Gruss, A 2011, 'Using heme as an energy boost for lactic acid bacteria', *Current Opinion in Biotechnology*, vol. 22, no. 2, pp. 143-149.

Leslie, SB, Israeli, E, Lighthart, B, Crowe, JH & Crowe, LM 1995, 'Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying', *Applied and Environmental Microbiology*, vol. 61, no. 10, pp. 3592-3597.

Lesuffleur, T, Barbat, A, Dussaulx, E & Zweibaum, A 1990, 'Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells', *Cancer Research*, vol. 50, no. 19, pp. 6334-6343.

Li, C, Nie, S-P, Ding, Q, Zhu, K-X, Wang, Z-J, Xiong, T, Gong, J & Xie, M-Y 2014, 'Cholesterol-lowering effect of *Lactobacillus plantarum* NCU116 in a hyperlipidaemic rat model', *Journal of Functional Foods*, vol. 8, pp. 340-347.

Li, F, Schmerberg, CM & Ji, Q 2009, 'Accelerated tryptic digestion of proteins in plasma for absolute quantitation using a protein internal standard by liquid chromatography/tandem mass spectrometry', *Rapid Communications in Mass Spectrometry*, vol. 23, no. 5, pp. 729-732.

Li, YB, Xu, QQ, Yang, CJ, Yang, X, Lv, L, Yin, CH, Liu, XL & Yan, H 2014, 'Effects of probiotics on the growth performance and intestinal micro flora of broiler chickens', *Pakistan Journal of Pharmaceutical Sciences*, vol. 27, no. 3, pp. 713-717.

Licandro-Seraut, H, Scornec, H, Pédron, T, Cavin, JF & Sansonetti, PJ 2014, 'Functional genomics of *Lactobacillus casei* establishment in the gut', *Proceedings of the National Academy of Sciences*, vol. 111, no. 30, pp. E3101-E3109.

Liebler, DC and Zimmerman, LJ 2013, 'Targeted quantitation of proteins by mass spectrometry', *Biochemistry*, vol. 52 no. 22, pp. 3797-3806.

Ling, E, Feldman, G, Portnoi, M, Dagan, R, Overweg, K, Mulholland, F, Chalifa-caspi, V, Wells, J & Mizrachi-nebenzahl, Y 2004, 'Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse', *Clinical & Experimental Immunology*, vol. 138, no. 2, pp. 290-298.

Liong, M-T 2008, 'Safety of probiotics: translocation and infection', *Nutrition Reviews*, vol. 66, no. 4, pp. 192-202.

Little, JW, Mount, DW & Yanisch-Perron, CR 1981, 'Purified lexA protein is a repressor of the recA and lexA genes', *Proceedings of the National Academy of Sciences*, vol. 78, no. 7, pp. 4199-4203.

Liu, M, Bayjanov, JR, Renckens, B, Nauta, A & Siezen, RJ 2010, 'The proteolytic system of lactic acid bacteria revisited: a genomic comparison', *BMC Genomics*, vol. 11, no. 1, p. 36.

Liu, W, Xi, X, Sudu, Q, Kwok, L, Guo, Z, Hou, Q, Menhe, B, Sun, T & Zhang, H 2015, 'High-throughput sequencing reveals microbial community diversity of Tibetan naturally fermented yak milk', *Annals of Microbiology*, vol. 65, no. 3, pp. 1741-1751.

Liu, W, Zheng, Y, Kwok, L-Y, Sun, Z, Zhang, J, Guo, Z, Hou, Q, Menhe, B & Zhang, H 2015, 'High-throughput sequencing for the detection of the bacterial and fungal diversity in Mongolian naturally fermented cow's milk in Russia', *Bmc Microbiology*, vol. 15, no. 1, pp. 385-385.

Ljungh, A & Wadstrom, T 2006, 'Lactic acid bacteria as probiotics', *Current Issues in Intestinal Microbiology*, vol. 7, no. 2, pp. 73-90.

Loguercio, C, Federico, A, Tuccillo, C, Terracciano, F, D'Auria, MV, De Simone, C & Blanco, CDV 2005, 'Beneficial effects of a probiotic VSL# 3 on parameters of liver dysfunction in chronic liver diseases', *Journal of Clinical Gastroenterology*, vol. 39, no. 6, pp. 540-543.

Lorca, G, Torino, MaI, de Valdez, GF & Ljungh, Å 2002, 'Lactobacilli express cell surface proteins which mediate binding of immobilized collagen and fibronectin', *Fems Microbiology Letters*, vol. 206, no. 1, pp. 31-37.

Lortal, S, Van Heijenoort, J, Gruber, K & Sleytr, UB 1992, 'S-layer of *Lactobacillus helveticus* ATCC 12046: isolation, chemical characterization and re-formation after extraction with lithium chloride', *Journal of General Microbiology*, vol. 138, no. 3, pp. 611-618.

Louesdon, S, Charlot-Rougé, S, Tourdot-Maréchal, R, Bouix, M & Béal, C 2015, 'Membrane fatty acid composition and fluidity are involved in the resistance to freezing of *Lactobacillus buchneri* R1102 and *Bifidobacterium longum* R0175', *Microbial Biotechnology*, vol. 8, no. 2, pp. 311-318.

Lüders, S, Fallet, C & Franco-Lara, E 2009a, 'Proteome analysis of the *Escherichia coli* heat shock response under steady-state conditions', *Proteome Science*, vol. 7, no. 1, p. 1.

Lüders, S, Fallet, C & Franco-Lara, E 2009b, 'Proteome analysis of the *Escherichia coli* heat shock response under steady-state conditions', *Proteome Science*, vol. 7, no. 1, p. 36.

Ma, J, Stoter, G, Verweij, J & Schellens, JH 1996, 'Comparison of ethanol plasma-protein precipitation with plasma ultrafiltration and trichloroacetic acid protein precipitation for the measurement of unbound platinum concentrations', *Cancer Chemotherapy and Pharmacology*, vol. 38, no. 4, pp. 391-394.

Machado, MC, López, CS, Heras, H & Rivas, EA 2004, 'Osmotic response in *Lactobacillus casei* ATCC 393: biochemical and biophysical characteristics of membrane', *Archives of Biochemistry and Biophysics*, vol. 422, no. 1, pp. 61-70.

Makarova, K, Slesarev, A, Wolf, Y, Sorokin, A, Mirkin, B, Koonin, E, Pavlov, A, Pavlova, N, Karamychev, V & Polouchine, N 2006, 'Comparative genomics of the lactic acid bacteria', *Proceedings of the National Academy of Sciences*, vol. 103, no. 42, pp. 15611-15616.

Mansilla, MC, Cybulski, LE, Albanesi, D & de Mendoza, D 2004, 'Control of membrane lipid fluidity by molecular thermosensors', *Journal of Bacteriology*, vol. 186, no. 20, pp. 6681-6688.

Marles-Wright, J & Lewis, RJ 2007, 'Stress responses of bacteria', *Current Opinion in Structural Biology*, vol. 17, no. 6, pp. 755-760.

Marteau, PR, de Vrese, M, Cellier, CJ & Schrezenmeir, J 2001, 'Protection from gastrointestinal diseases with the use of probiotics', *The American Journal of Clinical Nutrition*, vol. 73, no. 2, pp. 430s-436s.

Martens, L, Hermjakob, H, Jones, P, Adamski, M, Taylor, C, States, D, Gevaert, K, Vandekerckhove, J & Apweiler, R 2005, 'PRIDE: the proteomics identifications database', *Proteomics*, vol. 5, no. 13, pp. 3537-3545.

Martin, I, Wendt, D & Heberer, M 2004, 'The role of bioreactors in tissue engineering', *TRENDS in Biotechnology*, vol. 22, no. 2, pp. 80-86.

Martín, R, Sánchez, B, Urdaci, MC, Langella, P, Suárez, JE & Bermúdez-Humarán, LG 2015, 'Effect of iron on the probiotic properties of the vaginal isolate *Lactobacillus jensenii* CECT 4306', *Microbiology*, vol. 161, no. Pt 4, pp. 708-718.

Matsumoto, Y, Kaito, C, Morishita, D, Kurokawa, K & Sekimizu, K 2007, 'Regulation of exoprotein gene expression by the *Staphylococcus aureus* cvfB gene', *Infection and Immunity*, vol. 75, no. 4, pp. 1964-1972.

Mäyrä-Mäkinen, A & Bigret, M 1998, 'Industrial use and production of lactic acid bacteria', *Food Science and Technology-New York-Marcel Dekker-*, pp. 73-102.

McGavin, M, Krajewska-Pietrasik, D, Ryden, C & Höök, M 1993, 'Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity', *Infection and Immunity*, vol. 61, no. 6, pp. 2479-2485.

McLeod, A, Zagorec, M, Champomier-Vergès, M-C, Naterstad, K & Axelsson, L 2010, 'Primary metabolism in *Lactobacillus sakei* food isolates by proteomic analysis', *BMC Microbiology*, vol. 10, no. 1, p. 120.

McSweeney, P, Walsh, E, Fox, P, Cogan, T, Drinan, F & Castelo-Gonzalez, M 1994, 'A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality', *Irish Journal of Agricultural and Food Research*, pp. 183-192.

Meyrand, M, Guillot, A, Goin, M, Furlan, S, Armalyte, J, Kulakauskas, S, Cortes-Perez, NG, Thomas, G, Chat, S & Péchoux, C 2013, 'Surface proteome analysis of a natural isolate of *Lactococcus lactis* reveals the presence of pili able to bind human intestinal epithelial cells', *Molecular & Cellular Proteomics*, vol. 12, no. 12, pp. 3935-3947.

Mikov, MM, Stojančević, MP & Bojić, GM 2014, 'Probiotics as a Promising Treatment for Inflammatory Bowel Disease', *Hospital*, vol. 1, no. 1, pp. 52-60.

Miller, KA, Phillips, RS, Mrázek, J & Hoover, TR 2013, 'Salmonella utilizes D-glucosaminate via a mannose family phosphotransferase system permease and associated enzymes', *Journal of Bacteriology*, vol. 195, no. 18, pp. 4057-4066.

Mills, S, Stanton, C, Fitzgerald, GF & Ross, R 2011a, 'Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again', in *Microbial Cell Factories*, vol. 10, p. S19.

Mills, S, Stanton, C, Fitzgerald, GF & Ross, RP 2011b, 'Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again', *Microb Cell Fact*, vol. 10, no. Suppl 1, p. S19.

Mitchell, WJ, Reizer, J, Herring, C, Hoischen, C & Saier, M 1993, 'Identification of a phosphoenolpyruvate: fructose phosphotransferase system (fructose-1-phosphate forming) in *Listeria monocytogenes'*, *Journal of Bacteriology*, vol. 175, no. 9, pp. 2758-2761.

Mohammadi, M 2013, 'Probiotics and Cancer', *Journal of Biology*, vol. 2, no. 4, pp. 202-209.

Mohedano, ML, Amblar, M, De La Fuente, A, Wells, JM & López, P 2016, 'The response regulator YycF inhibits expression of the fatty acid biosynthesis repressor FabT in *Streptococcus pneumoniae*', *Frontiers in Microbiology*, vol. 7, p. 1326.

Mojgani, N, Hussaini, F & Vaseji, N 2015, 'Characterization of Indigenous *Lactobacillus* Strains for Probiotic Properties', *Jundishapur Journal of Microbiology*, vol. 8, no. 2.

Monedero, V, Mazé, A, Boël, G, Zuniga, M, Beaufils, S, Hartke, A & Deutscher, J 2007, 'The phosphotransferase system of *Lactobacillus casei*: regulation of carbon metabolism and connection to cold shock response', *Journal of Molecular Microbiology and Biotechnology*, vol. 12, no. 1-2, pp. 20-32.

Monedero, V, Revilla-Guarinos, A & Zúñiga, M. 2017 'Physiological role of two-component signal transduction systems in food-associated lactic acid bacteria', *Advances in Applied Microbiology, v*ol. 99, pp. 1-51.

Mooney, RA, Darst, SA & Landick, R 2005, 'Sigma and RNA polymerase: an on-again, off-again relationship?', *Molecular Cell*, vol. 20, no. 3, pp. 335-345.

Moradian, A, Kalli, A, Sweredoski, MJ & Hess, S 2014, 'The top-down, middle-down, and bottom-up mass spectrometry approaches for characterization of histone variants and their post-translational modifications', *Proteomics*, vol. 14, no. 4-5, pp. 489-497.

Morel-Deville, F, Fauvel, F & Morel, P 1998, 'Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei* Franqoise Morel-Deville,′ t Franck Fauvel ‵ and Patrice Morel ‵ t', *Microbiology*, vol. 144 no. 10, pp. 2873-2883.

Moreno, AT, Oliveira, MLS, Ho, PL, Vadesilho, CF, Palma, GM, Ferreira, JM, Ferreira, DM, Santos, SR, Martinez, MB & Miyaji, EN 2012, 'Cross-reactivity of antipneumococcal surface protein C (PspC) antibodies with different strains and evaluation of inhibition of human complement factor H and secretory IgA binding via PspC', *Clinical and Vaccine Immunology*, vol. 19, no. 4, pp. 499-507.

Mori, H & Ito, K 2001, 'The Sec protein-translocation pathway', *Trends in Microbiology*, vol. 9, no. 10, pp. 494-500.

Morishita, T & Yajima, M 1995, 'Incomplete operation of biosynthetic and bioenergetic functions of the citric acid cycle in multiple auxotrophic lactobacilli', *Bioscience, Biotechnology, and Biochemistry*, vol. 59, no. 2, pp. 251-255.

Mozzi, F, Rollan, G, De Giori, GS & De Valdez, GF 2001, 'Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in *Lactobacillus casei* CRL 87', *Journal of Applied Microbiology*, vol. 91, no. 1, pp. 160-167.

Müller, J, Walter, F, van Dijl, JM & Behnke, D 1992, 'Suppression of the growth and export defects of an *Escherichia coli* secATs) mutant by a gene cloned from *Bacillus subtilis*', *Molecular and General Genetics MGG*, vol. 235, no. 1, pp. 89-96.

Müller, JP, Ozegowski, J, Vettermann, S, Swaving, J, Van Wely, K & Driessen, A 2000, 'Interaction of *Bacillus subtilis* CsaA with SecA and precursor proteins', *Biochemical Journal*, vol. 348, no. Pt 2, p. 367.

Müller, M, Reiß, S, Schlüter, R, Mäder, U, Beyer, A, Reiß, W, Marles-Wright, J, Lewis, RJ, Pförtner, H & Völker, U 2014, 'Deletion of membrane-associated Asp23 leads to upregulation of cell wall stress genes in *Staphylococcus aureus*', *Molecular Microbiology*, vol. 93, no. 6, pp. 1259-1268.

Munoz-Provencio, D, Perez-Martinez, G & Monedero, V 2011, 'Identification of surface Proteins from *Lactobacillus casei* BL23 able to bind fibronectin and collagen', *Probiotics and Antimicrobial Proteins*, vol. 3, no. 1, pp. 15-20.

Muñoz-Provencio, D, Pérez-Martínez, G & Monedero, V 2011, 'Identification of surface proteins from *Lactobacillus casei* BL23 able to bind fibronectin and collagen', *Probiotics and Antimicrobial Proteins*, vol. 3, no. 1, pp. 15-20.

Nagamachi, E, Shibuya, Si, Hirai, Y, Matsushita, O, Tomochika, Ki & Kanemasa, Y 1991, 'Adaptational changes of fatty acid composition and the physical state of membrane lipids following the change of growth temperature in *Yersinia enterocolitica*', *Microbiology and Immunology*, vol. 35, no. 12, pp. 1085-1093.

Nahar, A, Baker, AL, Bowman, JP & Britz, ML 2017, 'Draft genome sequences of two *Lactobacillus casei* strains isolated from Cheddar cheese and a fermented milk drink', *Genome Announcements*, vol. 5, no. 44, pp. e01235-01217.

Nancy, YY, Wagner, JR, Laird, MR, Melli, G, Rey, S, Lo, R, Dao, P, Sahinalp, SC, Ester, M & Foster, LJ 2010, 'PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes', *Bioinformatics*, vol. 26, no. 13, pp. 1608-1615.

Nannen, NL & Hutkins, RW 1991, 'Proton-translocating adenosine triphosphatase activity in lactic acid bacterial', *Journal of Dairy Science*, vol. 74, no. 3, pp. 747-751.

Narberhaus, F 2002, 'α-Crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network', *Microbiology and Molecular Biology Reviews*, vol. 66, no. 1, pp. 64-93.

Nezhad, MH, Knight, M & Britz, ML 2012, 'Evidence of changes in cell surface proteins during growth of *Lactobacillus casei* under acidic conditions', *Food Science and Biotechnology*, vol. 21, no. 1, pp. 253-260.

Nezhad, MH, Stenzel, D & Britz, M 2010, 'Effect of growth at low pH on the cell surface properties of a typical strain of *Lactobacillus casei* group', *Iranian Journal of Microbiology*, vol. 2, no. 3, p. 147.

Ng, SY, Koon, SS, Padam, BS & Chye, FY 2015, 'Evaluation of probiotic potential of lactic acid bacteria isolated from traditional Malaysian fermented Bambangan (Mangifera pajang)', *Cyta-Journal of Food*, vol. 13, no. 4, pp. 563-572.

Nie, NH, Bent, DH & Hull, CH 1970, *SPSS: Statistical package for the social sciences*, McGraw-Hill New York.

Nielsen, DS, Schillinger, U, Franz, CM, Bresciani, J, Amoa-Awua, W, Holzapfel, WH & Jakobsen, M 2007, '*Lactobacillus ghanensis* sp. nov., a motile lactic acid bacterium isolated from Ghanaian cocoa fermentations', *International Journal of Systematic and Evolutionary Microbiology*, vol. 57, no. 7, pp. 1468-1472.

Noh, SY, Kang, S-S, Yun, C-H & Han, SH 2015, 'Lipoteichoic acid from *Lactobacillus plantarum* inhibits Pam2CSK4-induced IL-8 production in human intestinal epithelial cells', *Molecular Immunology*, vol. 64, no. 1, pp. 183-189.

Nonin-Lecomte, S, Germain-Amiot, N, Gillet, R, Hallier, M, Ponchon, L, Dardel, F & Felden, B 2009, 'Ribosome hijacking: a role for small protein B during trans-translation', *EMBO Reports*, vol. 10, no. 2, pp. 160-165.

O'Farrell, PH 1975, 'High resolution two-dimensional electrophoresis of proteins', *Journal of Biological Chemistry*, vol. 250, no. 10, pp. 4007-4021.

Oakley, BB, Lillehoj, HS, Kogut, MH, Kim, WK, Maurer, JJ, Pedroso, A, Lee, MD, Collett, SR, Johnson, TJ & Cox, NA 2014, 'The chicken gastrointestinal microbiome', *Fems Microbiology Letters*, vol. 360, no. 2, pp. 100-112.

Olaya-Abril, A, Gómez-Gascón, L, Jiménez-Munguía, I, Obando, I & Rodríguez-Ortega, MJ 2012, 'Another turn of the screw in shaving Gram-positive bacteria: Optimization of proteomics surface protein identification in *Streptococcus pneumoniae*', *Journal of Proteomics*, vol. 75, no. 12, pp. 3733-3746.

Olaya-Abril, A, Jiménez-Munguía, I, Gómez-Gascón, L, Obando, I & Rodríguez-Ortega, MJ 2013, 'Identification of potential new protein vaccine candidates through pan-surfomic analysis of pneumococcal clinical isolates from adults', *PLoS ONE*, vol. 8, no. 7, p. e70365.

Olaya-Abril, A, Jiménez-Munguía, I, Gómez-Gascón, L & Rodríguez-Ortega, MJ 2014, 'Surfomics: shaving live organisms for a fast proteomic identification of surface proteins', *Journal of Proteomics*, vol. 97, pp. 164-176.

Olguín, N, Champomier-Vergès, M, Anglade, P, Baraige, F, Cordero-Otero, R, Bordons, A, Zagorec, M & Reguant, C 2015, 'Transcriptomic and proteomic analysis of *Oenococcus oeni* PSU-1 response to ethanol shock', *Food Microbiology*, vol. 51, pp. 87-95.

Oliveira, AS, Lima, JA, Rezende, CM & Pinto, AC 2009, 'Cyclopentenyl acids from sapucainha oil (*Carpotroche brasiliensis* Endl, *Flacourtiaceae*): the first antileprotic used in Brazil', *Química Nova*, vol. 32, no. 1, pp. 139-145.

Ooi, L-G & Liong, M-T 2010, 'Cholesterol-lowering effects of probiotics and prebiotics: a review of in vivo and in vitro findings', *International Journal of Molecular Sciences*, vol. 11, no. 6, pp. 2499-2522.

Österberg, S, Peso-Santos, Td & Shingler, V 2011, 'Regulation of alternative sigma factor use', *Annual Review of Microbiology*, vol. 65, pp. 37-55.

Palumbo, E, Favier, CF, Deghorain, M, Cocconcelli, PS, Grangette, C, Mercenier, A, Vaughan, EE & Hols, P 2004, 'Knockout of the alanine racemase gene in *Lactobacillus plantarum* results in septation defects and cell wall perforation', *Fems Microbiology Letters*, vol. 233, no. 1, pp. 131-138.

Pancholi, V & Chhatwal, GS 2003, 'Housekeeping enzymes as virulence factors for pathogens', *International Journal of Medical Microbiology*, vol. 293, no. 6, pp. 391-401.

Papadimitriou, K, Alegría, Á, Bron, PA, de Angelis, M, Gobbetti, M, Kleerebezem, M, Lemos, JA, Linares, DM, Ross, P & Stanton, C 2016, 'Stress Physiology of Lactic Acid Bacteria', *Microbiology and Molecular Biology Reviews*, vol. 80, no. 3, pp. 837-890.

Park, YU, Kim, MD, Jung, DH, Seo, DH, Jung, JH, Park, JG, Hong, SY, Cho, JY, Park, SY, Park, JW, Shin, WC & Park, CS 2015, 'Probiotic properties of lactic acid bacteria isolated from Korean rice wine Makgeolli', *Food Science and Biotechnology*, vol. 24, no. 5, pp. 1761-1766.

Parsons, JB, Broussard, TC, Bose, JL, Rosch, JW, Jackson, P, Subramanian, C & Rock, CO 2014, 'Identification of a two-component fatty acid kinase responsible for host fatty acid incorporation by *Staphylococcus aureus*', *Proceedings of the National Academy of Sciences*, vol. 111, no. 29, pp. 10532-10537.

Parvez, S, Malik, KA, Ah Kang, S & Kim, HY 2006, 'Probiotics and their fermented food products are beneficial for health', *Journal of Applied Microbiology*, vol. 100, no. 6, pp. 1171-1185.

Pedersen, MB, Gaudu, P, Lechardeur, D, Petit, M-A & Gruss, A 2012, 'Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology', *Annual Review of Food Science and Technology*, vol. 3, pp. 37-58.

Pelletier, C, Bouley, C, Cayuela, C, Bouttier, S, Bourlioux, P & Bellon-Fontaine, M-N 1997, 'Cell surface characteristics of *Lactobacillus casei* subsp. *casei, Lactobacillus paracasei* subsp. *paracasei,* and *Lactobacillus rhamnosus* strains', *Applied and Environmental Microbiology*, vol. 63, no. 5, pp. 1725-1731.

Pepinsky, RB 1991, 'Selective precipitation of proteins from guanidine hydrochloride-containing solutions with ethanol', *Analytical Biochemistry*, vol. 195, no. 1, pp. 177-181.

Pessione, A, Lamberti, C & Pessione, E 2010, 'Proteomics as a tool for studying energy metabolism in lactic acid bacteria', *Molecular BioSystems*, vol. 6, no. 8, pp. 1419-1430.

Petersen, TN, Brunak, S, von Heijne, G & Nielsen, H 2011, 'SignalP 4.0: discriminating signal peptides from transmembrane regions', *Nature Methods*, vol. 8, no. 10, pp. 785-786.

Peterson, S & Marshall, R 1990, 'Nonstarter lactobacilli in Cheddar cheese: a review', *Journal of Dairy Science*, vol. 73, no. 6, pp. 1395-1410.

Pettersen, EF, Goddard, TD, Huang, CC, Couch, GS, Greenblatt, DM, Meng, EC & Ferrin, TE 2004, 'UCSF Chimera - a visualization system for exploratory research and analysis', *Journal of Computational Chemistry*, vol. 25, no. 13, pp. 1605-1612.

Phan-Thanh, L & Mahouin, F 1999, 'A proteomic approach to study the acid response in *Listeria monocytogenes'*, *Electrophoresis*, vol. 20, no. 11, pp. 2214-2224.

Phillips, GJ & Silhavy, TJ 1990, 'Heat-shock proteins DnaK and GroEL facilitate export of LacZ hybrid proteins in *E. coli*'. *Nature*, vol. 344, no. 6269, p. 882.

Piatigorsky, J, O'Brien, WE, Norman, BL, Kalumuck, K, Wistow, GJ, Borras, T, Nickerson, JM & Wawrousek, EF 1988, 'Gene sharing by delta-crystallin and argininosuccinate lyase', *Proceedings of the National Academy of Sciences*, vol. 85, no. 10, pp. 3479-3483.

Piuri, M, Sanchez-Rivas, C & Ruzal, S 2003, 'Adaptation to high salt in *Lactobacillus*: role of peptides and proteolytic enzymes', *Journal of Applied Microbiology*, vol. 95, no. 2, pp. 372-379.

Piuri, M, Sanchez-Rivas, C & Ruzal, S 2005, 'Cell wall modifications during osmotic stress in *Lactobacillus casei*', *Journal of Applied Microbiology*, vol. 98, no. 1, pp. 84-95.

Polak-Berecka, M, Waśko, A, Paduch, R, Skrzypek, T & Sroka-Bartnicka, A 2014, 'The effect of cell surface components on adhesion ability of *Lactobacillus rhamnosus*', *Antonie Van Leeuwenhoek*, vol. 106, no. 4, pp. 751-762.

Poolman, B & Glaasker, E 1998, 'Regulation of compatible solute accumulation in bacteria', *Molecular Microbiology*, vol. 29, no. 2, pp. 397-407.

Postma, P, Lengeler, J & Jacobson, G 1993, 'Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria', *Microbiological Reviews*, vol. 57, no. 3, pp. 543-594.

Prágai, Z & Harwood, CR 2002, 'Regulatory interactions between the Pho and σ^B-dependent general stress regulons of *Bacillus subtilis*', *Microbiology*, vol. 148, no. 5, pp. 1593-1602.

Prasad, J, McJarrow, P & Gopal, P 2003, 'Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying', *Applied and Environmental Microbiology*, vol. 69, no. 2, pp. 917-925.

Price, CW, Fawcett, P, Cérémonie, H, Su, N, Murphy, CK & Youngman, P 2001, 'Genome-wide analysis of the general stress response in *Bacillus subtilis*', *Molecular Microbiology*, vol. 41, no. 4, pp. 757-774.

Prisciandaro, L, Geier, M, Butler, R, Cummins, A & Howarth, G 2009, 'Probiotics and their derivatives as treatments for inflammatory bowel disease', *Inflammatory Bowel Diseases*, vol. 15, no. 12, pp. 1906-1914.

Puglia, CD & Powell, SR 1984, 'Inhibition of cellular antioxidants: a possible mechanism of toxic cell injury', *Environmental Health Perspectives*, vol. 57, p. 307.

Rabatinová, A, Šanderová, H, Matějčková, JJ, Korelusová, J, Sojka, L, Barvík, I, Papoušková, V, Sklenář, V, Žídek, L & Krásný, L 2013, 'The δ subunit of RNA polymerase is required for rapid changes in gene expression and competitive fitness of the cell', *Journal of Bacteriology*, vol. 195, no. 11, pp. 2603-2611.

Raghunathan, K, Harris, PT, Spurbeck, RR, Arvidson, CG & Arvidson, DN 2014, 'Crystal structure of an efficacious gonococcal adherence inhibitor: An enolase from *Lactobacillus gasseri*', *Febs Letters*, vol. 588, no. 14, pp. 2212-2216.

Ramiah, K, van Reenen, CA & Dicks, LM 2008, 'Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive

exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*', *Research in Microbiology*, vol. 159, no. 6, pp. 470-475.

Randall, LL 1992, 'Peptide binding by chaperone SecB: implications for recognition of nonnative structure', *Science*, vol. 257, no. 5067, pp. 241-245.

Regulski, K, Courtin, P, Meyrand, M, Claes, IJ, Lebeer, S, Vanderleyden, J, Hols, P, Guillot, A & Chapot-Chartier, M-P 2012, 'Analysis of the peptidoglycan hydrolase complement of *Lactobacillus casei* and characterization of the major γ-D-glutamyl-L-lysyl-endopeptidase', *PLoS ONE*, vol. 7, no. 2, p. e32301.

Reid, G 1999, 'The scientific basis for probiotic strains of *Lactobacillus*', *Applied and Environmental Microbiology*, vol. 65, no. 9, pp. 3763-3766.

Reid, G 2015, 'The growth potential for dairy probiotics', *International Dairy Journal*, vol. 49, pp. 16-22.

Reiff, C & Kelly, D 2010, 'Inflammatory bowel disease, gut bacteria and probiotic therapy', *International Journal of Medical Microbiology*, vol. 300, no. 1, pp. 25-33.

Richter, K, Haslbeck, M & Buchner, J 2010, 'The heat shock response: life on the verge of death', *Molecular Cell*, vol. 40, no. 2, pp. 253-266.

Rimaux, T, Rivière, A, Illeghems, K, Weckx, S, De Vuyst, L & Leroy, F 2012, 'Expression of the arginine deiminase pathway genes in *Lactobacillus sakei* is strain dependent and is affected by the environmental pH', *Applied and Environmental Microbiology*, vol. 78, no. 14, pp. 4874-4883.

Rimaux, T, Vrancken, G, Pothakos, V, Maes, D, De Vuyst, L & Leroy, F 2011, 'The kinetics of the arginine deiminase pathway in the meat starter culture *Lactobacillus sakei* CTC 494 are pH-dependent', *Food Mcrobiology*, vol. 28, no. 3, pp. 597-604.

Rivas-Sendra, A, Landete, J, Alcántara, C & Zúñiga, M 2011, 'Response of *Lactobacillus casei* BL23 to phenolic compounds', *Journal of Applied Microbiology*, vol. 111, no. 6, pp. 1473-1481.

Robinson, RK 1995, *A colour guide to cheese and fermented milks*, Chapman & Hall Ltd.

Rochat, T, Gratadoux, J-J, Gruss, A, Corthier, G, Maguin, E, Langella, P & van de Guchte, M 2006, 'Production of a heterologous nonheme catalase by *Lactobacillus casei*: an efficient tool for removal of H2O2 and protection of *Lactobacillus bulgaricus* from oxidative stress in milk', *Applied and Environmental Microbiology*, vol. 72, no. 8, pp. 5143-5149.

Rodríguez-Ortega, MJ, Norais, N, Bensi, G, Liberatori, S, Capo, S, Mora, M, Scarselli, M, Doro, F, Ferrari, G & Garaguso, I 2006, 'Characterization and identification of vaccine candidate proteins through analysis of the group A *Streptococcus* surface proteome', *Nature Biotechnology*, vol. 24, no. 2, p. 191.

Ross, R, Fitzgerald, G, Collins, K & Stanton, C 2002, 'Cheese delivering biocultures--probiotic cheese', *Australian Journal of Dairy Technology*, vol. 57, no. 2, p. 71.

Rossi, F, Zotta, T, Iacumin, L & Reale, A 2016, 'Theoretical insight into the heat shock response (HSR) regulation in *Lactobacillus casei* and *L. rhamnosus*', *Journal of Theoretical Biology*, vol. 402, pp. 21-37.

Rothman, JE & Schekman, R 2011, 'Molecular mechanism of protein folding in the cell', *Cell*, vol. 146, no. 6, pp. 851-854.

Rotilio, D, Della Corte, A, D'Imperio, M, Coletta, W, Marcone, S, Silvestri, C, Giordano, L, Di Michele, M & Donati, MB 2012, 'Proteomics: bases for protein complexity understanding', *Thrombosis Research*, vol. 129, no. 3, pp. 257-262.

Ruas-Madiedo, P, Gueimonde, M, Margolles, A, de los Losreyes-Gavilán, CG & Salminen, S 2006, 'Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus', *Journal of Food Protection*, vol. 69, no. 8, pp. 2011-2015.

Russell, N, Evans, R, Ter Steeg, P, Hellemons, J, Verheul, A & Abee, T 1995, 'Membranes as a target for stress adaptation', *International Journal of Food Microbiology*, vol. 28, no. 2, pp. 255-261.

Russo, P, De La Luz Mohedano, M, Capozzi, V, De Palencia, PF, López, P, Spano, G & Fiocco, D 2012, 'Comparative proteomic analysis of *Lactobacillus plantarum* WCFS1 and ΔctsR mutant strains under physiological and heat stress conditions', *International Journal of Molecular Sciences*, vol. 13, no. 9, pp. 10680-10696.

Saarela, M, Mogensen, G, Fonden, R, Mättö, J & Mattila-Sandholm, T 2000, 'Probiotic bacteria: safety, functional and technological properties', *Journal of Biotechnology*, vol. 84, no. 3, pp. 197-215.

Saier Jr, MH 2015, 'The bacterial phosphotransferase system: new frontiers 50 years after its discovery', *Journal of Molecular Microbiology and Biotechnology*, vol. 25, no. 2-3, pp. 73-78.

Saier, M, Ye, J-J, Klinke, S & Nino, E 1996, 'Identification of an anaerobically induced phosphoenolpyruvate-dependent fructose-specific phosphotransferase system and evidence

for the Embden-Meyerhof glycolytic pathway in the heterofermentative bacterium *Lactobacillus brevis*', *Journal of Bacteriology*, vol. 178, no. 1, pp. 314-316.

Saini, G 2010, *Bacterial hydrophobicity: assessment techniques, applications and extension to colloids*, Oregon State University.

Sala, A, Bordes, P & Genevaux, P 2014, 'Multitasking SecB chaperones in bacteria', *Frontiers in Microbiology*, vol. 5.

Salar-Behzadi, S, Wu, S, Toegel, S, Hofrichter, M, Altenburger, I, Unger, FM, Wirth, M & Viernstein, H 2013, 'Impact of heat treatment and spray drying on cellular properties and culturability of *Bifidobacterium bifidum* BB-12', *Food Research International*, vol. 54, no. 1, pp. 93-101.

Salzillo, M, Vastano, V, Capri, U, Muscariello, L, Sacco, M & Marasco, R 2015, 'Identification and characterization of enolase as a collagen-binding protein in *Lactobacillus plantarum*', *Journal of Basic Microbiology*, vol. 55, no. 7, pp. 890-897.

Sanchez, B, Bressollier, P & Urdaci, MC 2008, 'Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host', *FEMS Immunology & Medical Microbiology*, vol. 54, no. 1, pp. 1-17.

Sánchez, B, Burns, P, Ruiz, L, Binetti, A, Vinderola, G, Reinheimer, J, Margolles, A, Ruas-Madiedo, P & Clara, G 2013, 'Co-culture affects protein profile and heat tolerance of *Lactobacillus deLrueckii* subsp. *lactis* and *Bifidobacterium longum*', *Food Research International*, vol. 54, no. 1, pp. 1080-1083.

Sanders, JW, Venema, G & Kok, J 1999, 'Environmental stress responses in *Lactococcus lactis*', *FEMS Microbiology Reviews*, vol. 23, no. 4, pp. 483-501.

Santivarangkna, C, Kulozik, U & Foerst, P 2007, 'Alternative drying processes for the industrial preservation of lactic acid starter cultures', *Biotechnology Progress*, vol. 23, no. 2, pp. 302-315.

Santos, MS 1996, 'Biogenic amines: their importance in foods', *International Journal of Food Microbiology*, vol. 29, no. 2-3, pp. 213-231.

Saraniya, A & Jeevaratnam, K 2015, 'In vitro probiotic evaluation of phytase producing *Lactobacillus* species isolated from Uttapam batter and their application in soy milk fermentation', *Journal of Food Science and Technology-Mysore*, vol. 52, no. 9, pp. 5631-5640.

Savijoki, K, Ingmer, H & Varmanen, P 2006, 'Proteolytic systems of lactic acid bacteria', *Applied Microbiology and Biotechnology*, vol. 71, no. 4, pp. 394-406.

Scatassa, ML, Gaglio, R, Macaluso, G, Francesca, N, Randazzo, W, Cardamone, C, Di Grigoli, A, Moschetti, G & Settanni, L 2015, 'Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses', *Food Microbiology*, vol. 52, pp. 31-41.

Schmidt, A, Schiesswohl, M, Völker, U, Hecker, M & Schumann, W 1992, 'Cloning, sequencing, mapping, and transcriptional analysis of the *groESL* operon from *Bacillus subtilis*', *Journal of Bacteriology*, vol. 174, no. 12, pp. 3993-3999.

Schmidt, G, Hertel, C & Hammes, WP 1999, 'Molecular characterisation of the *dnaK* operon of *Lactobacillus sakei* LTH681', *Systematic and Applied Microbiology*, vol. 22, no. 3, pp. 321-328.

Schmid, S, Bevilacqua, C, & Crutz-Le Coq, AM 2012, 'Alternative sigma factor σ H activates competence gene expression in *Lactobacillus sakei*. *BMC microbiology*, vol. 12, no.1, pp 32.

Schneewind, O & Missiakas, D 2014, 'Sec-secretion and sortase-mediated anchoring of proteins in Gram-positive bacteria', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, vol. 1843, no. 8, pp. 1687-1697.

Schneewind, O & Missiakas, DM 2012, 'Protein secretion and surface display in Gram-positive bacteria', *Phil. Trans. R. Soc. B*, vol. 367, no. 1592, pp. 1123-1139.

Schott, A-S, Behr, J, Quinn, J & Vogel, RF 2016, 'MALDI-TOF Mass Spectrometry Enables a Comprehensive and Fast Analysis of Dynamics and Qualities of Stress Responses of *Lactobacillus paracasei* subsp. *paracasei* F19', *PloS One*, vol. 11, no. 10, p. e0165504.

Schott, A-S, Behr, Jr, Geißler, AJ, Kuster, B, Hahne, H & Vogel, RF 2017, 'Quantitative Proteomics for the Comprehensive Analysis of Stress Responses of *Lactobacillus paracasei* subsp. *paracasei* F19', *Journal of Proteome Research*, vol. 16, no. 10, pp. 3816-3829.

Schulz, A & Schumann, W 1996, 'hrcA, the first gene of the *Bacillus subtilis dnaK* operon encodes a negative regulator of class I heat shock genes', *Journal of Bacteriology*, vol. 178, no. 4, pp. 1088-1093.

Schumacher, MA, Hengst, CD, Bush, MJ, Le, T, Tran, NT, Chandra, G, Zeng, W, Travis, B, Brennan, RG & Buttner, MJ 2018, 'The MerR-like protein BldC binds DNA direct repeats as cooperative multimers to regulate Streptomyces development', *Nature Communications*, vol. 9, no. 1, p. 1139.

Schumann, W 2016, 'Regulation of bacterial heat shock stimulons', *Cell Stress and Chaperones*, vol. 21, no. 6, pp. 959-968.

Schumann, W, Hecker, M & Msadek, T 2002, 'Regulation and Function of Heat-lnducible Genes in *Bacillus subtilis*', in *Bacillus subtilis* and Its Closest Relatives, *American Society of Microbiology*, pp. 359-368.

Scott, JR & Barnett, TC 2006, 'Surface proteins of Gram-positive bacteria and how they get there', *Annu. Rev. Microbiol.*, vol. 60, pp. 397-423.

Šeme, H, Gjuračić, K, Kos, B, Fujs, Š, Štempelj, M, Petković, H, Šušković, J, Bogovič Matijašić, B & Kosec, G 2014, 'Acid resistance and response to pH-induced stress in two *Lactobacillus plantarum* strains with probiotic potential', *Beneficial Microbes*, vol. 6, no. 3, pp. 369-379.

Sengupta, R, Altermann, E, Anderson, RC, McNabb, WC, Moughan, PJ & Roy, NC 2013, 'The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract', *Mediators of Inflammation*, vol. 2013.

Serrazanetti, DI, Gianotti, A, Montanari, C & Gottardi, D 2013, *Dynamic Stresses of Lactic Acid Bacteria Associated to Fermentation Processes*, INTECH Open Access Publisher.

Servant, P, Grandvalet, C & Mazodier, P 2000, 'The RheA repressor is the thermosensor of the HSP18 heat shock response in *Streptomyces albus*', *Proceedings of the National Academy of Sciences*, vol. 97, no. 7, pp. 3538-3543.

Seth, A, Yan, F, Polk, DB & Rao, R 2008, 'Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC-and MAP kinase-dependent mechanism', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 294, no. 4, pp. G1060-G1069.

Settanni, L, Franciosi, E, Cavazza, A, Cocconcelli, PS & Poznanski, E 2011, 'Extension of Tosèla cheese shelf-life using non-starter lactic acid bacteria', *Food Microbiology*, vol. 28, no. 5, pp. 883-890.

Settanni, L & Moschetti, G 2010, 'Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits', *Food Microbiology*, vol. 27, no. 6, pp. 691-697.

Shen, H-B & Chou, K-C 2009, 'Gpos-mPLoc: a top-down approach to improve the quality of predicting subcellular localization of Gram-positive bacterial proteins', *Protein and Peptide Letters*, vol. 16, no. 12, pp. 1478-1484.

Shida, K, Kiyoshima-Shibata, J, Kaji, R, Nagaoka, M & Nanno, M 2009, 'Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms', *Immunology*, vol. 128, no. 1pt2, pp. e858-e869.

Shimizu, K 1993, 'An overview on the control system design of bioreactors', *Measurement and Control*, pp. 65-84.

Silhavy, TJ, Kahne, D & Walker, S 2010, 'The bacterial cell envelope', *Cold Spring Harbor Perspectives in Biology*, vol. 2, no. 5, p. a000414.

Silva, J, Carvalho, AS, Teixera, P & Gibbs, P 2005, 'Effect of stress on cells of *Lactobacillus deLrueckeii* spp. *bulgaricus*', *Journal of Food Technology*, vol. 3, pp. 479-490.

Smit, G, Smit, BA & Engels, WJ 2005, 'Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products', *FEMS Microbiology Reviews*, vol. 29, no. 3, pp. 591-610.

Smits, HH, Engering, A, van der Kleij, D, de Jong, EC, Schipper, K, van Capel, TM, Zaat, BA, Yazdanbakhsh, M, Wierenga, EA & van Kooyk, Y 2005, 'Selective probiotic bacteria induce IL-10–producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin', *Journal of Allergy and Clinical Immunology*, vol. 115, no. 6, pp. 1260-1267.

Sofu, A, Sayilgan, E & Guney, G 2015, 'Experimental design for removal of Fe (II) and Zn (II) Ions by different lactic acid bacteria Bibomasses', *International Journal of Environmental Research*, vol. 9, no. 1, pp. 93-100.

Solis, N & Cordwell, SJ 2011, 'Current methodologies for proteomics of bacterial surface-exposed and cell envelope proteins', *Proteomics*, vol. 11, no. 15, pp. 3169-3189.

Solis, N, Larsen, MR & Cordwell, SJ 2010, 'Improved accuracy of cell surface shaving proteomics in *Staphylococcus aureus* using a false-positive control', *Proteomics*, vol. 10, no. 10, pp. 2037-2049.

Soriano, G, Sánchez, E & Guarner, C 2012, '[Probiotics in liver diseases]', *Nutricion hospitalaria*, vol. 28, no. 3, pp. 558-563.

Sosa-Castaneda, J, Hernandez-Mendoza, A, Astiazaran-Garcia, H, Garcia, HS, Estrada-Montoya, MC, Gonzalez-Cordova, AF & Vallejo-Cordoba, B 2015, 'Screening of *Lactobacillus* strains for their ability to produce conjugated linoleic acid in milk and to adhere to the intestinal tract', *Journal of Dairy Science*, vol. 98, no. 10, pp. 6651-6659.

Stadlbauer, V, Mookerjee, RP, Hodges, S, Wright, GA, Davies, NA & Jalan, R 2008, 'Effect of probiotic treatment on deranged neutrophil function and cytokine responses in patients with compensated alcoholic cirrhosis', *Journal of Hepatology*, vol. 48, no. 6, pp. 945-951.

Standish, AJ & Morona, R 2014, 'The role of bacterial protein tyrosine phosphatases in the regulation of the biosynthesis of secreted polysaccharides', *Antioxidants & Redox Signaling*, vol. 20, no. 14, pp. 2274-2289.

Stiles, ME & Holzapfel, WH 1997, 'Lactic acid bacteria of foods and their current taxonomy', *International Journal of Food Microbiology*, vol. 36, no. 1, pp. 1-29.

Stock, AM, Robinson, VL & Goudreau, PN 2000, 'Two-component signal transduction', *Annual Review of Biochemistry*, vol. 69, no. 1, pp. 183-215.

Stoller, G, Rücknagel, KP, Nierhaus, KH, Schmid, FX, Fischer, G & Rahfeld, J-U 1995, 'A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor', *The EMBO Journal*, vol. 14, no. 20, p. 4939.

Straus, D, Walter, W & Gross, CA 1990, 'DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of sigma 32', *Genes & Development*, vol. 4, no. 12a, pp. 2202-2209.

Streit, F, Delettre, J, Corrieu, G & Béal, C 2008, 'Acid adaptation of *Lactobacillus deLrueckii* subsp. *bulgaricus* induces physiological responses at membrane and cytosolic levels that improves cryotolerance', *Journal of Applied Microbiology*, vol. 105, no. 4, pp. 1071-1080.

Štyriak, I, Nemcova, R, Chang, YH & Ljungh, Å 2003, 'Binding of extracellular matrix molecules by probiotic bacteria', *Letters in Applied Microbiology*, vol. 37, no. 4, pp. 329-333.

Suarez, FL, Savaiano, DA & Levitt, MD 1995, 'A comparison of symptoms after the consumption of milk or lactose-hydrolyzed milk by people with self-reported severe lactose intolerance', *New England Journal of Medicine*, vol. 333, no. 1, pp. 1-4.

Sugimoto, S & Sonomoto, K 2008, 'Molecular chaperones in lactic acid bacteria: physiological consequences and biochemical properties', *Journal of Bioscience and Bioengineering*, vol. 106, no. 4, pp. 324-336.

Suokko, A, Poutanen, M, Savijoki, K, Kalkkinen, N & Varmanen, P 2008, 'ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*', *Proteomics*, vol. 8, no. 5, pp. 1029-1041.

Suokko, A, Savijoki, K, Malinen, E, Palva, A & Varmanen, P 2005, 'Characterization of a mobile *clpL* gene from *Lactobacillus rhamnosus*', *Applied and Environmental Microbiology*, vol. 71, no. 4, pp. 2061-2069.

Suutari, M & Laakso, S 1992, 'Temperature adaptation in *Lactobacillus fermentum*: interconversions of oleic, vaccenic and dihydrosterulic acids', *Microbiology*, vol. 138, no. 3, pp. 445-450.

Suzuki, H, Ikeda, A, Tsuchimoto, S, Adachi, K-i, Noguchi, A, Fukumori, Y & Kanemori, M 2012, 'Synergistic Binding of DnaJ and DnaK Chaperones to Heat Shock Transcription Factor σ^{32} Ensures Its Characteristic High Metabolic Instability implications for heat shock protein 70 (Hsp70)-Hsp40 mode of function', *Journal of Biological Chemistry*, vol. 287, no. 23, pp. 19275-19283.

Szajewska, H & Mrukowicz, JZ 2005, 'Use of probiotics in children with acute diarrhea', *Pediatric Drugs*, vol. 7, no. 2, pp. 111-122.

Tatusov, RL, Galperin, MY, Natale, DA & Koonin, EV 2000, 'The COG database: a tool for genome-scale analysis of protein functions and evolution', *Nucleic Acids Research*, vol. 28, no. 1, pp. 33-36.

Teixeira, P, Castro, H, Mohácsi-Farkas, C & Kirby, R 1997, 'Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress', *Journal of Applied Microbiology*, vol. 83, no. 2, pp. 219-226.

Tiong, HK, Hartson, S & Muriana, PM 2015, 'Comparison of five methods for direct extraction of surface proteins from *Listeria monocytogenes* for proteomic analysis by orbitrap mass spectrometry', *Journal of Microbiological Methods*, vol. 110, pp. 54-60.

Tjalsma, H, Lambooy, L, Hermans, PW & Swinkels, DW 2008, 'Shedding & shaving: disclosure of proteomic expressions on a bacterial face', *Proteomics*, vol. 8, no. 7, pp. 1415-1428.

Toba, T, Virkola, R, Westerlund, B, Bjorkman, Y, Sillanpaa, J, Vartio, T, Kalkkinen, N & Korhonen, TK 1995, 'A collagen-binding S-layer protein in *Lactobacillus crispatus*', *Applied and Environmental Microbiology*, vol. 61, no. 7, pp. 2467-2471.

Tomb, J-F, White, O, Kerlavage, AR, Clayton, RA, Sutton, GG, Fleischmann, RD, Ketchum, KA, Klenk, HP, Gill, S & Dougherty, BA 1997, 'The complete genome sequence of the gastric pathogen *Helicobacter pylori*', *Nature*, vol. 388, no. 6642, pp. 539-547.

Tong, Y, Zhai, Q, Wang, G, Zhang, Q, Liu, X, Tian, F, Zhao, J, Zhang, H & Chen, W 2017, 'System-wide analysis of manganese starvation-induced metabolism in key elements of *Lactobacillus plantarum*', *RSC Advances*, vol. 7, no. 21, pp. 12959-12968.

Tripathy, S, Padhi, S, Sen, R, Mohanty, S, Samanta, M & Maiti, N 2016, 'Profiling of *Brevibacillus borstelensis* transcriptome exposed to high temperature shock', *Genomics*, vol. 107, no. 1, pp. 33-39.

Tuohy, KM, Probert, HM, Smejkal, CW & Gibson, GR 2003, 'Using probiotics and prebiotics to improve gut health', *Drug Discovery Today*, vol. 8, no. 15, pp. 692-700.

Turujman, N & Durr, IF 1975, 'Decarboxylation of glyoxylate by a pyruvate oxidase in *Lactobacillus plantarum*', *International Journal of Biochemistry*, vol. 6, no. 9, pp. 643-648.

Ueta, M, Ohniwa, RL, Yoshida, H, Maki, Y, Wada, C & Wada, A 2008, 'Role of HPF (hibernation promoting factor) in translational activity in *Escherichia coli*', *Journal of Biochemistry*, vol. 143, no. 3, pp. 425-433.

Ullers, RS, Ang, D, Schwager, F, Georgopoulos, C & Genevaux, P 2007, 'Trigger Factor can antagonize both SecB and DnaK/DnaJ chaperone functions in *Escherichia coli*', *Proceedings of the National Academy of Sciences*, vol. 104, no. 9, pp. 3101-3106.

van Bokhorst-van de Veen, H, Abee, T, Tempelaars, M, Bron, PA, Kleerebezem, M & Marco, ML 2011, 'Short-and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*', *Applied and Environmental Microbiology*, vol. 77, no. 15, pp. 5247-5256.

van de Guchte, M, Serror, P, Chervaux, C, Smokvina, T, Ehrlich, SD & Maguin, E 2002, 'Stress responses in lactic acid bacteria', *Antonie Van Leeuwenhoek*, vol. 82, no. 1-4, pp. 187-216.

Van Loosdrecht, M, Lyklema, J, Norde, W, Schraa, G & Zehnder, A 1987, 'The role of bacterial cell wall hydrophobicity in adhesion', *Applied and Environmental Microbiology*, vol. 53, no. 8, pp. 1893-1897.

Vaughan, EE, de Vries, MC, Zoetendal, EG, Ben-Amor, K, Akkermans, AD & de Vos, WM 2002, 'The intestinal LABs', in *Lactic Acid Bacteria: Genetics, Metabolism and Applications*, Springer, pp. 341-352.

Veiga, P, Piquet, S, Maisons, A, Furlan, S, Courtin, P, Chapot-Chartier, MP & Kulakauskas, S 2006, 'Identification of an essential gene responsible for d-Asp incorporation in the *Lactococcus lactis* peptidoglycan crossbridge', *Molecular Microbiology*, vol. 62, no. 6, pp. 1713-1724.

Ventura, M, Canchaya, C, Zink, R, Fitzgerald, GF & Van Sinderen, D 2004, 'Characterization of the *groEL* and *groES* loci in *Bifidobacterium breve* UCC 2003: genetic, transcriptional, and phylogenetic analyses', *Applied and Environmental Microbiology*, vol. 70, no. 10, pp. 6197-6209.

Ventura, M, Jankovic, I, Walker, DC, Pridmore, RD & Zink, R 2002, 'Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*', *Applied and Environmental Microbiology*, vol. 68, no. 12, pp. 6172-6181.

Versteeg, S, Escher, A, Wende, A, Wiegert, T & Schumann, W 2003, 'Regulation of the *Bacillus subtilis* heat shock gene *htpG* is under positive control', *Journal of Bacteriology*, vol. 185, no. 2, pp. 466-474.

Veyrat, A, Monedero, V & Perez-Martinez, G 1994, 'Glucose transport by the phosphoenolpyruvate: mannose phosphotransferase system in *Lactobacillus casei* ATCC 393 and its role in carbon catabolite repression', *Microbiology*, vol. 140, no. 5, pp. 1141-1149.

Vido, K, Diemer, H, Van Dorsselaer, A, Leize, E, Juillard, V, Gruss, A & Gaudu, P 2005, 'Roles of thioredoxin reductase during the aerobic life of *Lactococcus lactis*', *Journal of Bacteriology*, vol. 187, no. 2, pp. 601-610.

Villena, J, Oliveira, MLS, Ferreira, PC, Salva, S & Alvarez, S 2011, 'Lactic acid bacteria in the prevention of pneumococcal respiratory infection: future opportunities and challenges', *International Immunopharmacology*, vol. 11, no. 11, pp. 1633-1645.

Vizcaíno, JA, Csordas, A, Del-Toro, N, Dianes, JA, Griss, J, Lavidas, I, Mayer, G, Perez-Riverol, Y, Reisinger, F & Ternent, T 2015, '2016 update of the PRIDE database and its related tools', *Nucleic Acids Research*, vol. 44, no. D1, pp. D447-D456.

Vonk, RJ, Reckman, GA, Harmsen, HJ & Priebe, MG 2012, *Probiotics and Lactose Intolerance*, INTECH Open Access Publisher.

Waddington, L, Cyr, T, Hefford, M, Hansen, LT & Kalmokoff, M 2010, 'Understanding the acid tolerance response of bifidobacteria', *Journal of Applied Microbiology*, vol. 108, no. 4, pp. 1408-1420.

Wahlström, E, Vitikainen, M, Kontinen, VP & Sarvas, M 2003, 'The extracytoplasmic folding factor PrsA is required for protein secretion only in the presence of the cell wall in *Bacillus subtilis*', *Microbiology*, vol. 149, no. 3, pp. 569-577.

Walker, DC, Girgis, HS & Klaenhammer, TR 1999, 'The groESL Chaperone Operon of *Lactobacillus johnsonii*', *Applied and Environmental Microbiology*, vol. 65, no. 7, pp. 3033-3041.

Walters, MS & Mobley, HL 2009, 'Identification of uropathogenic *Escherichia coli* surface proteins by shotgun proteomics', *Journal of Microbiological Methods*, vol. 78, no. 2, pp. 131-135.

Wang, B, Wei, H, Yuan, J, Li, Q, Li, Y, Li, N & Li, J 2008, 'Identification of a surface protein from *Lactobacillus reuteri* JCM1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells', *Current Microbiology*, vol. 57, no. 1, pp. 33-38.

Wang, G, Xia, Y, Cui, J, Gu, Z, Song, Y, Chen, YQ, Chen, H, Zhang, H & Chen, W 2013, 'The roles of moonlighting proteins in bacteria', *Current Issues in Molelular Biology*, vol. 16, pp. 15-22.

Wang, R, Jiang, L, Zhang, M, Zhao, L, Hao, Y, Guo, H, Sang, Y, Zhang, H & Ren, F 2017, 'The Adhesion of *Lactobacillus salivarius* REN to a Human Intestinal Epithelial Cell Line Requires S-layer Proteins', *Scientific Reports*, vol. 7, p. 44029.

Wang, XF, Tian, F, Cao, RM, Li, J, Wu, SM, Guo, XK & Chen, TX 2015, 'Antimicrobial activity of human beta-defensins against lactic acid bacteria', *Natural Product Research*, vol. 29, no. 22, pp. 2164-2166.

Wang, Y, Xie, J, Li, Y, Dong, S, Liu, H, Chen, J, Wang, Y, Zhao, S, Zhang, Y & Zhang, H 2016, 'Probiotic *Lactobacillus casei* Zhang reduces pro-inflammatory cytokine production and hepatic inflammation in a rat model of acute liver failure', *European Journal of Nutrition*, vol. 55, no. 2, pp. 821-831.

Warner, JB & Lolkema, JS 2003, 'CcpA-dependent carbon catabolite repression in bacteria', *Microbiology and Molecular Biology Reviews*, vol. 67, no. 4, pp. 475-490.

Wasko, A, Polak-Berecka, M, Paduch, R & Jozwiak, K 2014, 'The effect of moonlighting proteins on the adhesion and aggregation ability of *Lactobacillus helveticus*', *Anaerobe*, vol. 30, pp. 161-168.

Weidmann, S, Maitre, M, Laurent, J, Coucheney, F, Rieu, A & Guzzo, J 2017, 'Production of the small heat shock protein Lo18 from *Oenococcus oeni* in *Lactococcus lactis* improves its stress tolerance', *Int J Food Microbiol*., no. 247, pp. 18-23.

Weiss, A & Shaw, LN 2015, 'Small things considered: the small accessory subunits of RNA polymerase in Gram-positive bacteria', *FEMS Microbiology Reviews*, vol. 39, no. 4, pp. 541-554.

West, AH & Stock, AM 2001, 'Histidine kinases and response regulator proteins in two-component signaling systems', *Trends in Biochemical Sciences*, vol. 26, no. 6, pp. 369-376.

Whiteford, DC, Klingelhoets, JJ, Bambenek, MH & Dahl, JL 2011, 'Deletion of the histone-like protein (Hlp) from *Mycobacterium smegmatis* results in increased sensitivity to UV exposure, freezing and isoniazid', *Microbiology*, vol. 157, no. 2, pp. 327-335.

Wild, J, Rossmeissl, P, Walter, WA & Gross, CA 1996, 'Involvement of the DnaK-DnaJ-GrpE chaperone team in protein secretion in *Escherichia coli*', *Journal of Bacteriology*, vol. 178, no. 12, pp. 3608-3613.

Wilkins, JC, Homer, KA & Beighton, D 2001, 'Altered protein expression of *Streptococcus oralis* cultured at low pH revealed by two-dimensional gel electrophoresis', *Applied and Environmental Microbiology*, vol. 67, no. 8, pp. 3396-3405.

Wilkins, MR, Appel, RD, Van Eyk, JE, Chung, M, Görg, A, Hecker, M, Huber, LA, Langen, H, Link, AJ & Paik, YK 2006, 'Guidelines for the next 10 years of proteomics', *Proteomics*, vol. 6, no. 1, pp. 4-8.

Wilkins, MR, Pasquali, C, Appel, RD, Ou, K, Golaz, O, Sanchez, J-C, Yan, JX, Gooley, AA, Hughes, G & Humphery-Smith, I 1996, 'From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and arnino acid analysis', *Nature Biotechnology*, vol. 14, no. 1, p. 61.

Wilson, R, Diseberg, AF, Gordon, L, Zivkovic, S, Tatarczuch, L, Mackie, EJ, Gorman, JJ & Bateman, JF 2010, 'Comprehensive profiling of cartilage extracellular matrix formation and maturation using sequential extraction and label-free quantitative proteomics', *Molecular & Cellular Proteomics*, vol. 9, no. 6, pp. 1296-1313.

Wolfe, AJ 2015, 'Glycolysis for the microbiome generation', *Microbiology Spectrum*, vol. 3, no. 3.

Wu, C, Tran, JC, Zamdborg, L, Durbin, KR, Li, M, Ahlf, DR, Early, BP, Thomas, PM, Sweedler, JV & Kelleher, NL 2012, 'A protease for 'middle-down' proteomics', *Nature Methods*, vol. 9, no. 8, pp. 822-824.

Wu, C, Zhang, J, Chen, W, Wang, M, Du, G & Chen, J 2012, 'A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance', *Applied Microbiology and Biotechnology*, vol. 93, no. 2, pp. 707-722.

Wu, D, He, J, Gong, Y, Chen, D, Zhu, X, Qiu, N, Sun, M, Li, M & Yu, Z 2011, 'Proteomic analysis reveals the strategies of *Bacillus thuringiensis* YBT-1520 for survival under long-term heat stress', *Proteomics*, vol. 11, no. 13, pp. 2580-2591.

Wu, J & Rosen, B 1991, 'The ArsR protein is a trans-acting regulatory protein', *Molecular Microbiology*, vol. 5, no. 6, pp. 1331-1336.

Wu, Z, Pan, D, Guo, Y & Zeng, X 2015, 'N-acetylmuramic acid triggers anti-inflammatory capacity in LPS-induced RAW 264.7 cells and mice', *Journal of Functional Foods*, vol. 13, pp. 108-116.

Xolalpa, W, Vallecillo, AJ, Lara, M, Mendoza-Hernandez, G, Comini, M, Spallek, R, Singh, M & Espitia, C 2007, 'Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*', *Proteomics*, vol. 7, no. 18, pp. 3332-3341.

Xu, H, Caimano, MJ, Lin, T, He, M, Radolf, JD, Norris, SJ, Gheradini, F, Wolfe, AJ & Yang, XF 2010, 'Role of acetyl-phosphate in activation of the Rrp2-RpoN-RpoS pathway in *Borrelia burgdorferi*', *PLoS Pathogens*, vol. 6, no. 9, p. e1001104.

Yan, F, Cao, H, Cover, TL, Whitehead, R, Washington, MK & Polk, DB 2007, 'Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth', *Gastroenterology*, vol. 132, no. 2, pp. 562-575.

Yan, F & Polk, DB 2002, 'Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells', *Journal of Biological Chemistry*, vol. 277, no. 52, pp. 50959-50965.

Yan, F & Polk, DB 2012, '*Lactobacillus rhamnosus* GG: an updated strategy to use microbial products to promote health', *Functional Food Reviews (Print)*, vol. 4, no. 2, p. 77.

Yan, S & Wu, G 2015, 'Large-scale evolutionary analyses on SecB subunits of bacterial Sec system', *PloS One*, vol. 10, no. 3, p. e0120417.

Yates III, JR 2004, 'Mass spectral analysis in proteomics', *Annu. Rev. Biophys. Biomol. Struct.*, vol. 33, pp. 297-316.

Yu, J, Hui, W, Cao, C, Pan, L, Zhang, H & Zhang, W 2018, 'Integrative Genomic and Proteomic Analysis of the Response of *Lactobacillus casei* Zhang to Glucose Restriction', *Journal of Proteome Research*, vol. 17, no. 3, pp. 1290-1299.

Yu, NY, Wagner, JR, Laird, MR, Melli, G, Rey, S, Lo, R, Dao, P, Sahinalp, SC, Ester, M & Foster, LJ 2010, 'PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes', *Bioinformatics*, vol. 26, no. 13, pp. 1608-1615.

Zhai, Z, Douillard, FP, An, H, Wang, G, Guo, X, Luo, Y & Hao, Y 2014, 'Proteomic characterization of the acid tolerance response in *Lactobacillus deLrueckii* subsp. *bulgaricus* CAUH1 and functional identification of a novel acid stress-related transcriptional regulator Ldb0677', *Environmental Microbiology*, vol. 16, no. 6, pp. 1524-1537.

Zhang, W, Cao, C, Zhang, J, Kwok, LY, Zhang, H, Chen, Y 2018, '*Lactobacillus casei* asp23 gene contributes to gentamycin resistance via regulating specific membrane-associated proteins', *J Dairy Sci*. vol. 101, no. 3, pp. 1915-20.

Zhang, CX, Creskey, MC, Cyr, TD, Brooks, B, Huang, H, Pagotto, F & Lin, M 2013, 'Proteomic identification of *Listeria monocytogenes* surface-associated proteins', *Proteomics*, vol. 13, no. 20, pp. 3040-3045.

Zhang, WM, Wang, HF, Gao, K, Wang, C, Liu, L & Liu, JX 2015, '*Lactobacillus reuteri* glyceraldehyde-3-phosphate dehydrogenase functions in adhesion to intestinal epithelial cells', *Canadian Journal of Microbiology*, vol. 61, no. 5, pp. 373-380.

Zhang, Y-M & Rock, CO 2008, 'Membrane lipid homeostasis in bacteria', *Nature Reviews Microbiology*, vol. 6, no. 3, pp. 222-233.

Zhang, Y, Fonslow, BR, Shan, B, Baek, M-C & Yates III, JR 2013, 'Protein analysis by shotgun/bottom-up proteomics', *Chemical Reviews*, vol. 113, no. 4, pp. 2343-2394.

Zhou, F, Zhao, H, Zheng, J, Dziugan, P, Li, S & Zhang, B 2015, 'Evaluation of probiotic properties of *Lactobacillus* strains isolated from traditional Chinese cheese', *Annals of Microbiology*, vol. 65, no. 3, pp. 1419-1426.

Zhou, H, Ning, Z, E. Starr, A, Abu-Farha, M & Figeys, D 2011, 'Advancements in top-down proteomics', *Analytical Chemistry*, vol. 84, no. 2, pp. 720-734.

Zhou, J & Xu, Z 2005, 'The structural view of bacterial translocation-specific chaperone SecB: implications for function', *Molecular Microbiology*, vol. 58, no. 2, pp. 349-357.

Zhu, D, Sun, Y, Liu, F, Li, A, Yang, L & Meng, X-C 2016, 'Identification of surface-associated proteins of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 by enzymatic shaving', *Journal of Dairy Science*, vol. 99, no. 7, pp. 5155-5172.

Zhu, L, Xu, X, Wang, L, Dong, H, Yu, B & Ma, Y 2015, 'NADP$^+$-preferring D-lactate dehydrogenase from *SporoLactobacillus inulinus*', *Applied and Environmental Microbiology*, vol. 81, no. 18, pp. 6294-6301.

Zúñiga, M, Champomier-Verges, M, Zagorec, M & Pérez-Martínez, G 1998, 'Structural and Functional Analysis of the Gene Cluster Encoding the Enzymes of the Arginine Deiminase Pathway of *Lactobacillus sakei*', *Journal of Bacteriology*, vol. 180, no. 16, pp. 4154-4159.

Zúñiga, M, Pérez, G & González-Candelas, F 2002, 'Evolution of arginine deiminase (ADI) pathway genes', *Molecular Phylogenetics and Evolution*, vol. 25, no. 3, pp. 429-444.

APPENDIX

Supplementary Table 3. 1. MaxQuant output files *peptides.txt* and *proteinGroups.txt* that summarise the complete sets of peptides and proteins, respectively, identified in *L. casei* GCRL163 samples.

Supplementary Table 3. 2. Proteins that meet the filtering criteria and statistical comparison of LFQ protein expression data for *L. casei* GCRL163 proteins at different prolonged heat stress.

Supplementary Table 3. 3. Mean-centred (Z-scored) LFQ expression data and functional annotation of *L. casei* GCRL163 proteins based on the result of T-profiler analysis.

Supplementary Table 3. 4. Filtered proteins based on proteins identified in at least 3 replicates, to identify proteins specific to temperatures in *L. casei* GCRL163.

Supplementary Table 4. 1. Validated list of protein fractions from LiCl-sucrose, trypsin shaving and ECF protein fractions at different growth temperatures.

Supplementary Table 4. 2. MaxQuant output files for LiCl-sucrose, trypsin shaving and ECF protein fractions.

Supplementary Table 4. 3. Cytoplasmic proteins considered as surface proteins by detection in at least two of the three protein fractions (LiCl-sucrose, trypsin shaving and ECF fractions).

Supplementary Table 4. 4. Total surface proteins including extra-cytoplasmic and cytoplasmic-surface-associated proteins identified in *L. casei* GCRL163 from the LiCl-sucrose, trypsin shaving and ECF protein fractions at different growth temperatures.

Supplementary Table 5. 1. Filtered proteins from LiCl-sucrose, trypsin shaving and extracellular cultures fluid extracts from *L. casei* GCRL163 at mid-exponential and stationary growth phases.

Supplementary Table 5. 2. Proteins common to mid-exponential and stationary growth phases in the LiCl-sucrose, trypsin shaving and extracellular cultures fluid extracts from *L. casei* GCRL163.

Supplementary Table 7. 1. Cell-free-extract proteins of *L. casei* GCRL163 showing abundant expression at different growth temperatures.